Induction of hair follicles using neonatal mouse dermis and human keratinocytes: relevance for improved burn wound treatments

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Presented in fulfilment for the degree of Master of Science in Medicine

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February 2020

DECLARATION

I, Thudzelani Takalani Austin Malise, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Acknowledgments

I want to say thanks to the following wonderful people:

I want to say thanks to my mother for believing in me and for being patient with me. Thank for your words encouragement and for always providing for me.

To my siblings, Ndiwavhudi and Mukonazwothe, for all the well wishes you gave me.

To Professor Sue Kidson, for affording me an environment to growth in both personal and academic aspects of my life. Your mentorship skills are well cherished. For guiding me and for helping me to develop skills to interrogate scientific reports to understand them better.

To Dr Robea Ballo, for your patience and for helping understand all the technical aspects of my dissertation and for all the extra hours you spent assisting throughout my experiments.

To Kidson Lab members: Dennis, for welcoming me to the lab and for sacrificing your precious time to help me set up my experiments. Dimakatso, for guidance in all aspects of life and for your educated advice. Thulisa, for making sure our orders arrive in time and making there is order in the lab. Sylvia, for all warm words of encouragement. Claire, for being there to assist in the lab.

To Desiree, thank you for helping me to set up keratinocyte culture experiments.

To the Prince Lab, for welcoming me to your lab meetings, it was a wonderful experience.

To the CSIR Synthetic Biology group, Shivani, Jerolen, Brian, Tracey, Ezio, Dimakatso, specifically Janine ,for welcoming to your stimulating spaces.

Table of Contents

List of Figures

List of Tables

Abstract

Second degree burns result in the destruction of the skin as well as its adnexa. Following such burns the wound heals without the formation of skin appendages and hair follicles. In normal embryonic development hair follicle formation requires the interaction between epithelial (keratinocytes) and mesenchyme cells. Attempts to recapitulate the process of hair induction in wounded skin *in vitro* using human cells have to date been unsuccessful. The aim of this project is to attempt to elicit the early steps of hair follicle formation (induction) by co-culturing primary human keratinocytes with embryonic murine mesenchyme cells and assessing changes in expression patterns of genes associated with or reflective of induction.

Mesenchymal cells and keratinocytes cells were obtained by enzymatically digesting dorsal neonatal mouse skin and neonatal human foreskin using dispase and collagenase. Cells were cultured separately, and their growth dynamics measured. The isolated neonatal mouse mesenchymal cells were shown to have hair induction potential because they expressed dermal papilla signature genes, Alp, Sox2 and Vcan. However, this characteristic was lost during *in vitro* propagation suggesting that mesenchymal cells lose their hair inductive potential during culture. In contrast, when cultured at high densities or in spheroids in hanging drops, the dermal papilla signature genes were upregulated suggesting that this might be a way to maintain inductive potential.

Primary foreskin keratinocytes expressed high levels of basal layer marker, keratin 5 (K5), and low levels of the early differentiation marker, K10, suggesting that the isolated keratinocytes have stem cell properties. When co-cultured with neonatal mouse mesenchymal cells using Transwells, the mesenchymal cells were able to elicit colony formation on keratinocytes in co-cultures, indicating that they support keratinocyte proliferation.

It was not possible to do hair follicle induction marker analysis of human foreskin keratinocytes cocultures because of challenges and difficulties encountered during expansion. Therefore, Immortalised HaCaT keratinocytes were tested. HaCaT keratinocytes were shown to be induced during cocultures because they upregulated Wnt signalling genes, β-catenin and NF-KB. As an additional approach, human foreskin keratinocytes were cultured in medium containing Wnt signalling pathway ligand, Wnt3a. β-catenin and NF-KB were slightly reduced, and only Lef1 was upregulated in human foreskin keratinocytes cultured in Wnt3a conditioned medium. The results of this study show that neonatal mouse mesenchymal cells have hair inducing capabilities and it is lost by in vitro propagation and can be restored by spheroid cell culture. The results also demonstrate that human foreskin keratinocytes need to be expanded using efficient culture methods to maintain an undifferentiated state.

1 Chapter 1: Introduction, Literature Review and Aims

1.1 Introduction

According to World Health Organization (WHO), it costs South Africa an estimate of 26 million per year to take care of victims of burns caused by fuel fires predominantly in informal settlements (World Health Organization , 2018). The surviving victims are usually subjected to second- or third-degree burns, whereby the skin adnexa, including hair follicles are damaged to a point where they fail to regenerate. Currently, hair loss is treated by transplanting hair follicles from unaffected areas of the body, however, burn injuries usually affect the whole body, leaving an inadequate source of hair follicles for transplantation. In the case where there are no hair follicle donor sites, the available epidermal cells are expanded *in vitro* and transplanted to facilitate epithelialization of the wound (DeBruler, et al., 2018). To date there is no reliable method for regenerating hair follicles using somatic cells. For the skin to regenerate and form new hair follicles, a highly coordinated and precise inter-dependent epithelial-mesenchymal interaction is critical. However, the ability of *in vitro* propagated adult human epidermal cells to respond to human dermal signals for hair follicle formation has not been achieved (Zheng, et al., 2005; Ehama, et al., 2007; Kageyama, et al., 2018).

1.2 Skin structure

The skin is the outer covering of the body and acts as the first line of defence against the external environment. In addition to protection against environmental insults, the skin also regulates temperature via sweat and hair, and it is also an organ of sensations. The skin is made up of three main layers: the epidermis, which provides a protective barrier; the dermis, composed of collagen and blood capillaries, nerve endings and houses epithelial appendages including the hair follicles, sebaceous and sweat glands. Beneath the dermis is the hypodermis (subcutaneous tissue), a fat storage tissue which provides insulation (Fig. 1).

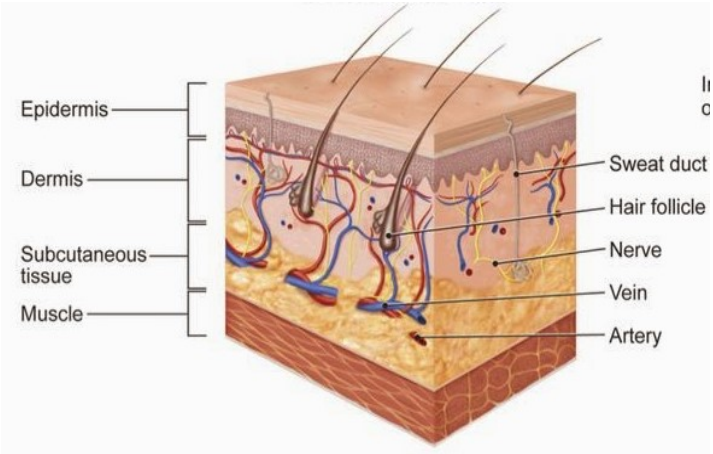


Figure 1: **Skin structure. Made up of three layers.** (1) The epidermis. Outermost layer. Provides a barrier between the outside environment. (2) The dermis. Lies beneath the epidermis. Contains connective tissue, hair follicles and sweat glands. (3) Subcutaneous tissue. Made up of fat and connective tissue. (Taken from **http://burnnoticeblog.blogspot.com/2015/04/diagnosisclassification-of-burns.html?m=1**).

1.2.1 Epidermis

The epidermis is a stratified squamous epithelium made up of the keratinocytes and melanocytes. The stratified epithelium is composed of the basal layer, the stratum spinosum, the stratum granulosum and the stratum corneum. The outermost layer (stratum corneum) is continuously shed off and replenished by cells from the basal layer. The basal layer rests on the basement membrane, a thin and dense sheet of specialised self-assembled extracellular matrix. The turnover of the epidermis is dependent on the keratinocyte stem cells that reside in the basal layer. Keratinocyte stem cells in the basal layer divide asymmetrically giving rise to one identical stem cell and a cell which starts to differentiate into transient amplifying cells. These detach from the basement membrane and further differentiate as they move up the epidermal layers. As they move up the epidermal layers, the DNA degrades, the cell becomes dehydrated and loses its nucleus. The keratinocytes become flattened and cornified providing structural strength for protection against environmental insults.

Each epidermal layer is unique and marks the level of differentiation of keratinocytes. They also undergo morphological and cytostructural changes. The undifferentiated keratinocytes in the in the basal layer express keratin 5 (K5) and 14 (K14). Terminally differentiating keratinocytes in the spinous layer lose K5 and K14, and express K10 and K1 (Fig. 2). As they further differentiate, they express involucrin, loricrin and profilaggrin in the granular layer and filaggrin in the cornified layer (Lee, et al., 2017). Keratins are cytoskeletal filament-forming proteins, they are water-insoluble, with a molecular weight ranging from 40-70 K. The family of 54 proteins are divided in two families, type I (acidic) and II (neutral or basic) and they can further be divided into three functional groups, simple keratins, barrier keratins and structural keratins. Simple keratins are expressed in embryonic and one-layered epithelia. The subunit composition of keratin filaments varies with cell type, period of embryonic development, stage of histological differentiation, cellular growth environment and disease state (Sun, et al., 1983). Barrier keratins are expressed in multi-layered squamous epithelia (Haines & Lane, 2012). Structural keratins are the harder keratins that are found in hair and the nail. All keratins are responsible for shape changes of keratinocytes as they move up the layers (Simpson et al., 2012).

1.2.2 Dermis

The dermis is a thick connective tissue layer that lies below the epidermis that provides mechanical support to the epidermis. It resists deforming forces, has tensile strength and can resume to its original shape after encountering deforming forces. The dermis contains the nerve endings, lymph vessels, sweat glands, sebaceous glands, blood capillaries and hair follicles. Fibroblasts produce elastin and collagen, which are crucial for keeping the skin firm and elastic. These fibres are secreted into a gel-like ground substance consisting of water, small solutes and proteoglycans. In addition to fibroblasts the dermis also contains mast cells which are the protective immune cells of the skin. The dermis is divided into two layers, the upper thin papillary layer and the lower dense reticular layer. The papillary layer is made up of loose connective tissue and contain numerous fibroblasts and relatively little

collagen fibres forming a network. The reticular layer is composed of a dense network of collagen fibres and far fewer fibroblasts.

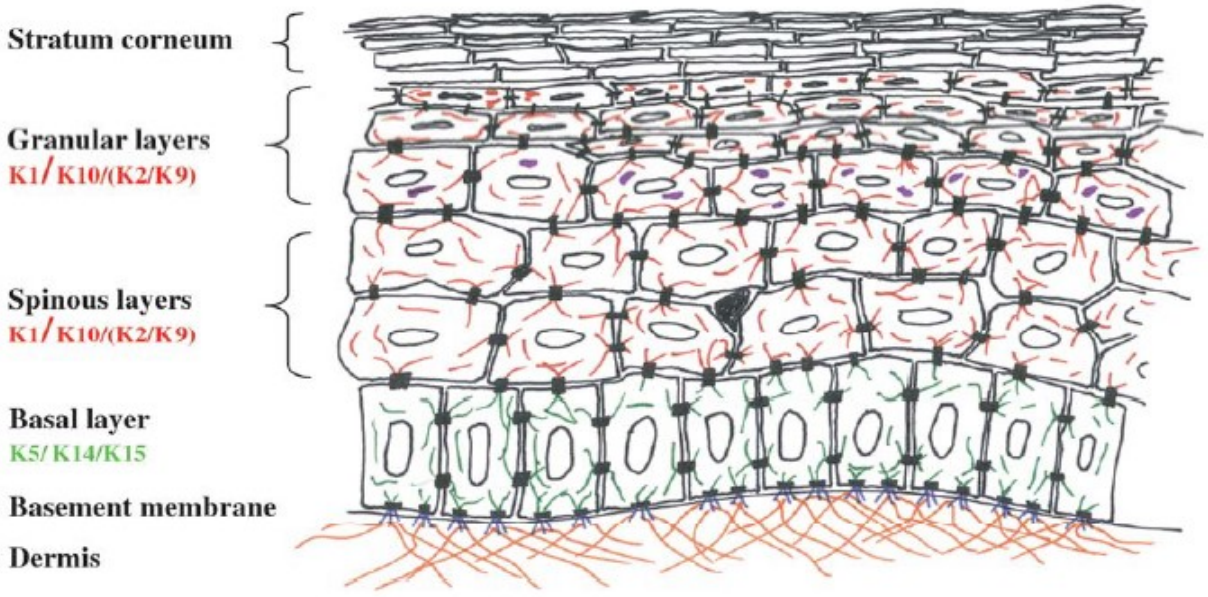


Figure 2: Keratin expression profile in the epidermis. (Taken from Kirfel et al., (2003))

1.2.3 The hair follicle

Each hair follicle (HF) can be considered as a mini-organ that is formed through epithelial-mesenchymal interactions that occur during embryogenesis (discussed below). Postnatally the hair follicle extends at an angle into the dermis and to a small extent to the hypodermis. At the base, the hair follicle epidermis forms an enlargement, called the bulb. The dermal papilla lies below the hair follicle and is surrounded by the epithelial bulb. The superior part of the hair follicle is made up of concentrically arranged epithelial layers including, the hair shaft, inner root and the outer root sheaths. The outer root sheath is continuous with the epidermis and the sebaceous gland. The bulge is an important cluster of cells located halfway down the hair shaft inferior to the sebaceous gland at the arrector pili muscle site on the outer connective sheath, and it is here that follicular stem cells are located. Beneath the concentric layers and on the edge of the dermal papilla in the bulb region of the hair follicle is the hair follicle matrix, also known as the secondary stem cell region of the hair follicle (Fig. 3). As opposed to the epidermis that express a limited range of keratins, the hair follicle expresses a diverse number of keratins in its concentric layers, with each layer expressing different combinations (Table. 1).

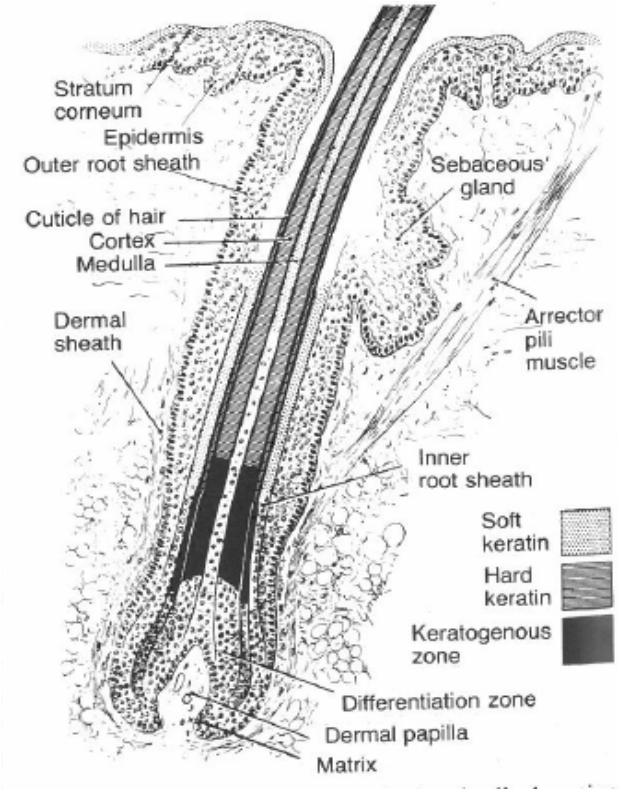


Figure 3: Schematic of a hair follicle showing three major layers. The outer root sheath, inner root sheath and the hair shaft (composed of cuticle of hair, cortex and medulla). (taken from Geneser (1986)).

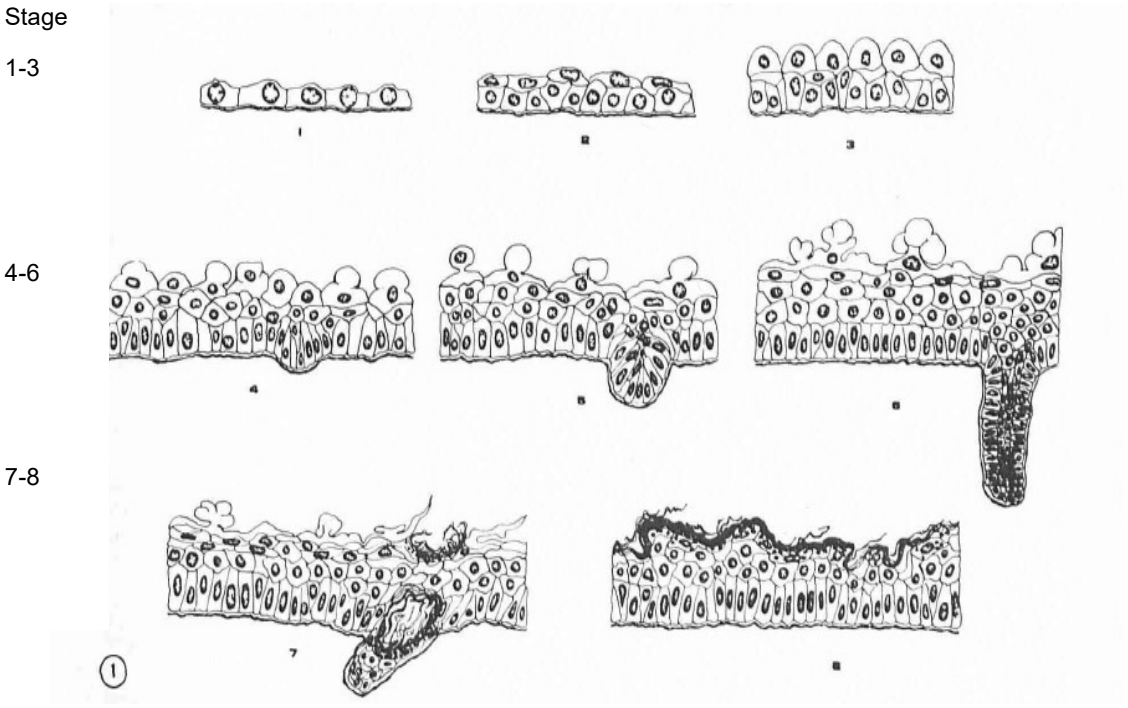
Table 1: The hair follicle keratin expression profile. Taken from Moll et al., (2008)

	Outer Root Sheath	**Companion layer**		**Inner Root Sheath**		**Hair shaft**
Basal layer	K5, 14, 15, 19	K6, 14, 16, 17, 75	**Henle**	K25, 27, 28, 71	**Medulla**	K6, 16, 17, 25, 27, 28, 33, 34, 37, 75, 81, 85
Suprbasal layer	K5, 6, 14, 16, 17		**Huxley**	K25, 27, 28, 74	**Cuticle**	K30, 32, 35, 40, 82, 85
			Cuticle	K25, 26, 27, 28, 71, 72, 73	**Matrix**	K35, 85
					Mid-upper cortex	K31, 33a, 33b, 34, 36, 37, 38, 39, 81, 83, 85, 86

1.3 Hair follicle morphogenesis

During embryogenesis the epidermis undergoes several steps starting with a simple single-layered epithelium which ultimately forms a stratified squamous keratinised epithelium together with hair follicles as well as other adnexa. The first steps in hair follicle induction involve localised thickening (placode) of the epithelium at around embryonic day 14.5 in mice (Tsai, et al., 2014) and between 65-95 days gestation age in humans (Fig. 4 A, stage 4) (Holbrook & Odland, 1975). Following placode formation, cells proliferate from the basal layer of the epidermis which results in the epidermal cells protruding downward into the mesoderm forming a hair germ (stage 5). Concurrently, the mesodermal cells start aggregating beneath the protruding hair germ (Fig.4 B, c). At stage 6 there is a further downgrowth and elongation of the hair peg and at this stage the hair follicle bulge can be observed. At stage 7 mature hair follicles can be seen on the surface of the skin, at this stage the hair follicles have hair shafts that extend from hair canals (Holbrook & Odland, 1975).

A



B

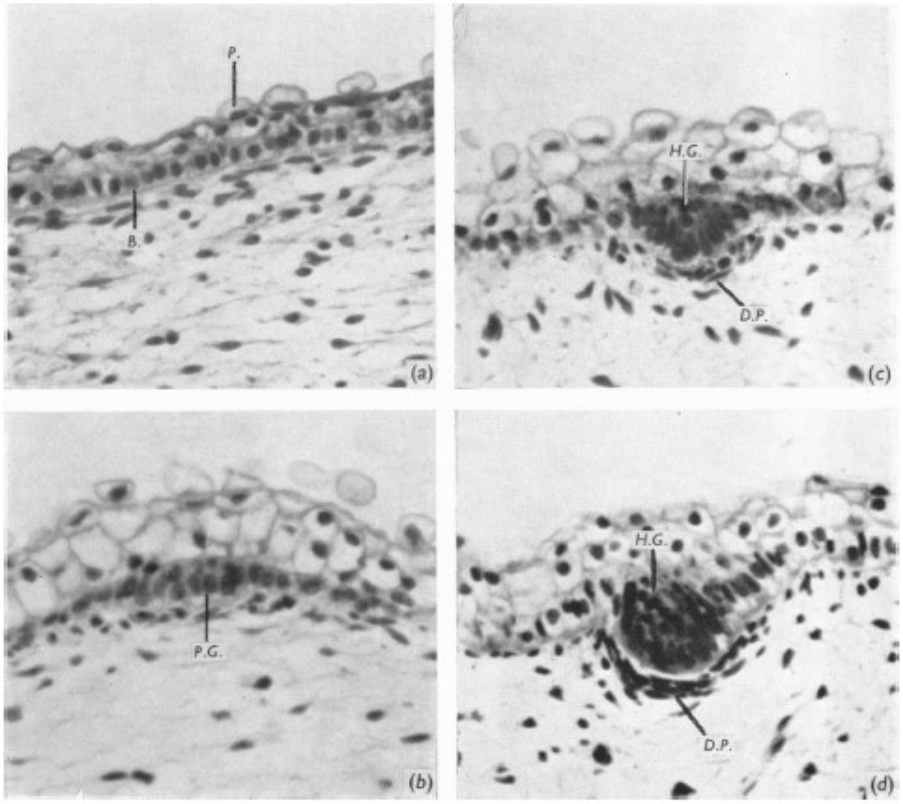


Figure 4: Hair follicle morphogenesis.

(A) **Human skin development stages.** Schematic diagram of the 8 stages of periderm development and epidermal differentiation. Corresponding estimated gestation ages for each stage (1) < 36 days, (2) 35-55 days, (3) 55-75 days, (4) 65-95 days, (5) 85-110 days, (6) 95-120 days, (7) 110-160 days, (8) > 160 days. (Taken from Holbrook et al (1975)).

(B) **Early hair follicle morphogenesis.** (a) Epidermis and mesenchyme before onset. B, basal layer of epidermal cells, P, Periderm. From arm skin of 11-week foetus. X480 (b) Pre-germ stage. Crowding of nuclei. From leg skin of 14-week foetus. x480. (c) Early hair germ (H.G) and anlage of dermal papilla (DP). From dorsal skin of 14-week foetus. x480. (d) A more advance hair germ and dermal papilla. From arm skin of 16-week foetus. x480. (taken from Breathnach and Smith (1968)).

1.4 Epithelial-mesenchymal interactions: role of Wnt and Eda signalling

As described above, hair follicle formation is initiated and maintained by the reciprocal interactions between epithelial and mesenchymal cells, a process that is tightly regulated by secreted signalling molecules. Among others the Wnt/β-catenin and the Eda/Edar/NF-Kβ signalling pathways have been shown to play a critical role in the communication of the epidermis and dermis during the initiation and progression of hair morphogenesis (Fig. 5 A) (reveiwed in Rishikaysh, et al., 2014). The other signalling pathways appear after the initiation of placode formation and they are less relevant to the work in this dissertation.

In the canonical Wnt pathway, secreted Wnt3a binds to frizzled and LRP5 or LRP6 receptor complex on the cell membrane. This results in the inhibition of glycogen synthase kinase-3 (GSK-3) which promotes the accumulation of β-catenin, which then translocates into the nucleus and together with TCF/LEF, induces the expression of Wnt target genes (Fig. 5 B) (Takahashi, et al., 2011).

To determine whether Wnt signalling is involved in hair placode formation previous investigators looked at the timing and expression pattern of Wnt signalling molecules during embryogenesis. Ridanpaa *et al*., (2001) performed β-catenin immunohistochemical staining in mouse whisker hair follicles. β-catenin was strongly expressed in the cytoplasm and membranes of hair placodes compared to the flanking basal epithelium at embryonic day 14.5 and 16.5, β-catenin protein staining was observed also in condensing mesenchymal cells. In the elongating hair peg and mature hair follicles β-catenin is strongly expressed in the outer root sheath, the bulge and the bulb region with some expression in the dermal papilla cells (Ridanpaa, et al., 2001).

To support the idea that Wnt signalling is involved in hair placode formation, Wnt activity was determined by using Beta-gal reporter mice that express ß-galactosidase under the control of Wnt-responsive multimerized LEF/TCF transcription factor binding sites. X-gal staining was used to detect β-galactosidase activity on whole-mount embryos and cryosections. X-gal staining was observed in hair placodes and dermal condensates at E14.5 and it was further observed in dermal papillae of elongating hair follicles and their surrounding epithelium from E15.5 to E18.5 (Tsai, et al., 2014). These observations show that Wnt/β-catenin is essential in some way during hair follicle development as it is expressed in both epithelial and mesenchymal cells all in the stages during hair follicle morphogenesis.

To determine whether Wnt/β-catenin signalling is crucial for hair follicle development Andl *et al.* (2002) blocked hair follicle development by utilising dickkopf-related protein 1 (Dkk1), a Wnt inhibitor. Dkk1 was ectopically expressed on the surface epithelium of a developing mouse embryo by placing its cDNA under the control of a K14 promoter. In situ hybridization was performed using Lef1 and β-catenin on skin tissue sections. Lef1 and β-catenin were absent In K14-Dkk1 transgenic mice skin sections (Andl, et al., 2002). In contrast, when mouse basal epithelial cells were forced to continuously

express a stabilized β-catenin, hair follicle development occurred early and the induced hair follicle placodes were actually larger than normal (Narhi, et al., 2008). This shows that Wnt/β-catenin signalling pathway is required for hair follicle cell fate determination and differentiation and its can be used when studying hair follicle morphogenesis progression.

Eda signalling is another pathway that has been shown to play a role during hair placode formation. The secreted ectodysplasin (Eda) binds to the TNF-receptor Edar on the cell membrane which results in the formation of a complex containing Edaradd, TRAF6, TAB2 and TAK1. TAK1 activates the IKK complex, which results in the ubiquitination and proteasomal degradation of the inhibitory proteins I k B and to the release of the NF-Kβ transcription factor. NF-Kβ translocates into the nucleus where it activates the transcription of target genes (Fig. 5 C) (Morlon, et al., 2006).

To determine whether Eda/Edar/NF-KB signalling is involved in the initiation of hair follicle placodes, Laurikkala et al., (2002), performed in situ hybridization in tissue sections and whole mount of mice back skin from embryonic day 11 to birth using Eda and Edar probes. Eda and Edar were co-expressed in the epidermis at embryonic day 12 and by embryonic day 14 and 15. Edar was shown to be restricted to hair placodes whereas Eda was expressed in the flanking epidermis. In new-borns both Eda and Edar were intensely expressed in the bulb region of the hair follicle, however, there was still expressed some Eda expression in the epidermis (Laurikkala, et al., 2002). The results suggest that Eda signalling is involved in placode formation as observed by the gene expression of Eda and Edar, respectively, in the epidermis and hair follicle placode.

To determine the role of Eda signalling during hair follicle induction Mustonen *et al*., (2003) used mice that overexpress Eda-A1 under the K14 promoter. The formed hair placodes were large. At embryonic day 15 the emerging hair follicles were already at stage 6 of hair follicle development. During embryonic development new hair follicle hair buds continued to emerge close to the previously formed ones and they occasionally were seen to fuse. In addition, at postnatal day 21 when the wild-type hair follicles were in telogen, the K14-Eda-A1 hair follicles were in anagen as visualised by hair bulbs that extend into the dermis. Furthermore, another interesting feature was the enlarged sebaceous glands (Mustonen, et al., 2003).

To gain further insight in the role of Eda-Edar signalling on placode formation, Mustonen *et al*., (2004) subsequently explored the role of Eda-Edar signalling by using exogeneous Eda. They cultured embryonic day 13 and 14 skin explants in the presence of conditioned medium harvested from Eda-A1 producing cells Cos7 cells and analysed the forming placodes by whole-mount in situ hybridization. Embryonic day 13 skin explants formed bigger and fused hair placodes within 24 hours. Embryonic day 14 skin explants showed no effects after 1 day in culture, however, after the third day hair placodes became bigger than control cultures (Mustonen, et al., 2004). Through these observations

it can be concluded that Eda signalling is crucial for the initiation of hair follicle placode and it regulates placode size.

To determine how Wnt and Eda signalling cooperate during placode formation previous studies have investigated this by manipulating the Wnt and Eda signalling components by making use of loss-of-function and gain-of-function models (Laurikkala, et al., 2002; Andl, et al., 2002; Zhang, et al., 2009).

To determine whether the Wnt signalling regulates Eda signalling Laurikkala et al., (2002) performed in situ hybridization for Eda and its receptor, Edar on Lef1 knockout mice skin tissue sections. There was no Eda gene expression on skin sections of embryonic day 15, 17 and postnatal day 0 as compared to wild-type mice skin sections where Eda is expressed on the epidermis. In contrast, Edar gene expression was observed in hair placodes of both wild-type and Lef1 knockout mice. These results suggest that Wnt signalling might regulate Eda/Edar/NF-KB signalling pathway by upregulating Eda expression on the skin epidermis.

Alternatively, to determine whether Wnt signalling lies upstream of Eda signalling Zhang et al., (2009) generated KRT5-rtTA;tetO-Dkk1 embryos in which epidermal Dkk1 expression can be induced by placing the pregnant mothers on oral doxycycline from embryonic day 0.5. In situ hybridization was performed in whole mount embryos using Eda, Edar and Wnt10b probes. Wnt10b is regulated by Eda signalling (Andl, et al., 2002) and it is expressed in hair placodes and in the follicular epithelial that lies adjacent the dermal condensate at the bulbous peg stage (Reddy, et al., 2001). There was no Edar and Wnt10b gene expression and Eda was not downregulated in hair placodes of embryonic day 14.5 KRT5-rtTA;tetO-Dkk1 embryos (Zhang, et al., 2009). The observations suggest that blocking of Wnt signalling ultimately results in a blocked Eda signalling.

To show that Wnt signalling is not regulated by Eda signalling Zhang et al., (2009) crossed TOPGAL (mice that carry transgenes containing 3 or 7 copies of a consensus LEF1/TCF DNA binding sequence), cond-lacZ (mice in which β-galactosidase reporter gene is regulated by the endogenous promoter of the conductin/axin2 gene,) and ßcatlacZ (B-catenin knock-in embryos) mice with Eda-/- (tabby) (Ta/Ta) and Edar-/- (downless) (dl/dl) mice, or mice with suppressed NF-KB activity (ΔN). X-gal staining of embryonic day 14.5 embryos showed that Wnt activity was present in the control, Eda-/- and Edar-/- embryos. In situ hybridization showed gene expression for β-catenin mRNA in sagittal skin sections of embryonic day 14.5 Edar-/- and ΔN embryos. However, the X-gal stained placodes were not well defined, and some were fused. This could suggest that Eda signalling is crucial for the fine tuning of Wnt signalling activity.

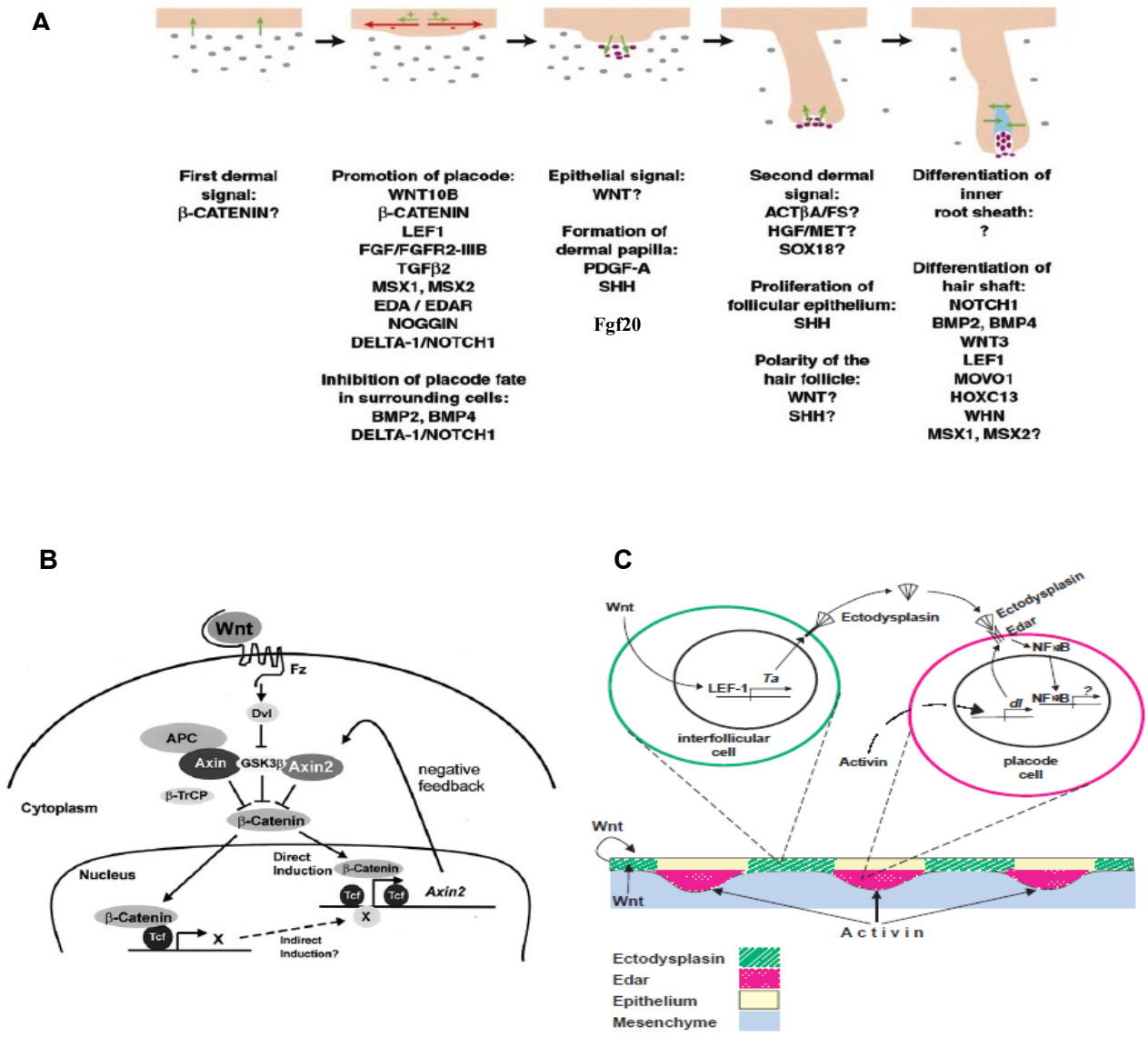


Figure 5: Wnt and Eda signalling.

(A) **Hair follicle development stages and signalling molecules.** The diagram show molecules that are important during hair follicle developmental stages. The representative coloured arrows indicate the direction of action of the signals. (Taken from Millar, (2002)).

(B) **Wnt signalling pathway.** Activated by binding of the Wnt protein to its receptor Frizzled resulting in signalling cascade that regulate transcription of downstream target genes. Upon stabilization of Wnt signals, β-catenin displaces the Groucho-related co-repressors and promotes Lymphoid enhancer-binding factor 1 (Lef1) a member of the T cell Factor (TCF)/LEF family of transcription factors to induce target genes. (Taken from Jho et al., (2002)).

(C) **Eda/Edar/NF-KB signalling.** This pathway gets activated through Lef1 which is regulated by the Wnt signalling pathway. The Lef1 induced Ectodysplasin (EDA) bind to its receptor EDAR and induces nuclear translocation of the transcription factor Nuclear Factor kappa beta (NF-KB) which activates the transcription of target genes. (Taken from Laurikkala et al., (2002)).

1.5 Dermal condensate formation: role of Fgf20 signalling

As described above, dermal cells start aggregating beneath the protruding hair germ forming what is known as the dermal condensate. Wnt signalling also plays a crucial role during dermal condensate formation as it was shown that β-catenin is active in both hair placodes and dermal condensates (Ridanpaa, et al., 2001). Recently, researchers have identified and shown Fgf20 a downstream target of the Wnt/β-catenin signalling pathway (Chamorro, et al., 2005), to be crucial during dermal condensate formation (Huh, et al., 2013).

To determine whether Fgf20 is required for dermal condensate formation Huh *et al.,* (2013), utilised $Fgf20^{\beta Gal/+}$ null (heterozygous) mice generated by replacing exon 1 of Fgf20 with a ß-galactosidase gene and homogenous mice $Fgf20^{\beta Gal/\beta Gal}$ generated by crossing $Fgf20^{\beta Gal/+}$ males with females. Both $Fgf20^{\beta Gal/+}$ and $Fgf20^{\beta Gal/\beta Gal}$ embryos formed hair placodes at E14.5, as observed by histology but at E16.5, $Fgf20^{\beta Gal/+}$ embryos had hair follicles at the peg stage and secondary hair follicles, on the contrary, $Fgf20^{\beta Gal/\beta Gal}$ embryos formed small hair follicles which were not associated with dermal condensations, though, there was some hair placodes that grew into the dermis forming hair pegs associated with a very small dermal condensation. Moreover, the hair follicles of $Fgf20^{\beta Gal/\beta Gal}$ embryos were bifurcated at E18.5 (Huh, et al., 2013). The results indicate that not only did mesenchymal condensate formation was reduced but the whole hair follicle development process was defected.

To provide the molecular evidence that dermal condensate formation was reduced in $Fgf20^{\beta Gal/\beta Gal}$ embryos they performed immunostaining for Sox2, a dermal condensate marker. Sox2 was expressed in dermal condensates of $Fgf20^{\beta Gal/+}$ embryos at E14.5 and not in $Fgf20^{\beta Gal/\beta Gal}$ embryos, even at E15.5. However, there was some little expression in dermal condensates of $Fgf20^{\beta Gal/\beta Gal}$ at E16.5 (Huh, et al., 2013). This further indicate that Fgf20 plays a critical role during dermal condensate formation.

To determine how Fgf20 facilitate dermal condensate formation, Biggs *et al.,* (2018), used 3D confocal microscopy to track dermal cells using a transgenic Sox-2-GFP. Sox2-GFP reporter whose expression correlates well with endogenous Sox2 was denser in the dermal condensate of $Fgf20^{\beta Gal/+}$ mouse tissue and less dense in the in interfollicular area of upper dermis and also in $Fgf20^{\beta Gal/\beta Gal}$ mouse tissue (Biggs, et al., 2018).

To quantify dermal condensate formation in more detail they analysed dermal condensation in 4 stages of placode development in $Fgf20^{\beta Gal/+}$ embryos. The number of Sox2 cells increased from a single layered placode stage, multi-layered placode, placode invagination stage to the stage where placodes have an anterior pocket where dermal condensates reside. Furthermore, Sox2 cells where getting closer to the placode from the first stage to the last stage of placode development. Even more, transmission electron microscopy and 3D confocal microscopy images revealed that dermal condensate cells change shape from spherical looking cells to elongated and convex shaped cells

during the placode developmental stages (Biggs, et al., 2018). This shows that Fgf20 signals regulate all the processes of dermal condensate formation, from migration, cell shape change to aggregation.

1.6 Hair follicle cycling

After birth and throughout life hair follicles undergo a growth cycle whereby hairs are renewed. The hair follicle growth cycle is divided into three phases, the active growth phase (anagen), regressing phase (catagen) and the resting phase (telogen) (Fig. 6).

During telogen the bulge lies atop the bulb region and on anagen onset the hair follicle stem cells in the bulge region proliferate and migrate to the bulb forming a secondary stem cell region the hair follicle matrix. Cells in the hair follicle matrix will then divide and differentiate to form new hair shaft and the inner root sheath (Morris, et al., 2004; Zhang, et al., 2009). In catagen the cells in the bulb region undergo apoptosis which results in the involution of the lower part of the hair follicle (Morris, et al., 2004; Zouboulis, et al., 2008).

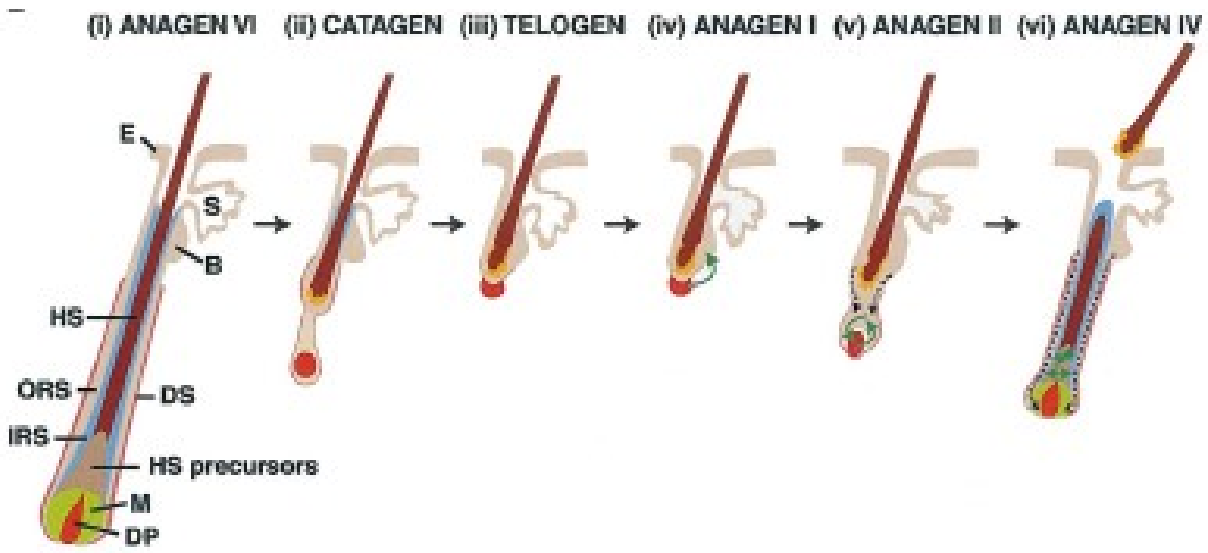


Figure 6: Hair growth cycle: (i) The active hair growth phase, (ii) Regression of the **hair follicle, (iii)** Resting stage. (Taken from Reddy et al., (2001))

1.7 Wounding

As discussed above, skin homeostasis is maintained by stem cell niches found in the interfollicular epidermis, the hair follicle bulge and the dermal papillae. Following an invasive injury, the skin loses its tensile strength and needs to be reconstructed by a process of wound healing, a natural restorative response which involves multiple cellular events. Stem cells in the skin systemically guarantee closure of the wound, and dead tissue is replaced by a vital tissue.

There are three main types of wound healing classified according to the time it takes for the skin barrier to be restored: primary healing, delayed primary healing and healing by secondary intention. A wound caused by a paper cut is so small that only a fraction of cellular constituents are damaged. The wound of this type will quickly heal because of the short distance at which keratinocytes and new blood vessels would have to travel. Delayed primary healing occurs when there is a delay in wound closer as observed in wounds that are contaminated or infected. The wound is first cleaned and then surgically closed, if it is not thoroughly cleaned chronic inflammation occurs resulting in prominent scarring. In secondary intention, for instance, following burns, the wound takes time to heal and there is an intense inflammatory reaction, a larger granulomatous tissue with a considerable tissue loss or the wound is extensive which make it difficult to bring the edges together. The wound is prone to scar formation and contractions. (reviewed in Swezey, 2014; Mercandetti, 2015).

A burn is an injury to the skin or any organic tissue that is primarily caused by thermal, electrical and chemical insults. Burns vary in severity and can be classified by how deep the injury is to the skin. In first degree burns (superficial), the damage is only on the outermost layer of the skin, the epidermis. First degree burns are characterised by pain, redness and mild swelling. In second degree (superficial partial) burns, the damage goes deep into the papillary region of the dermis. Second degree burns are characterised by pain, blisters, splotchy skin and severe swelling. The damage in third degree (Deep partial) burns involves the entire dermis. Third degree burns are characterised by a charred and insensate skin and eschar formation (Fig. 7) (reviewed in Kestrel Health Information, 2008).

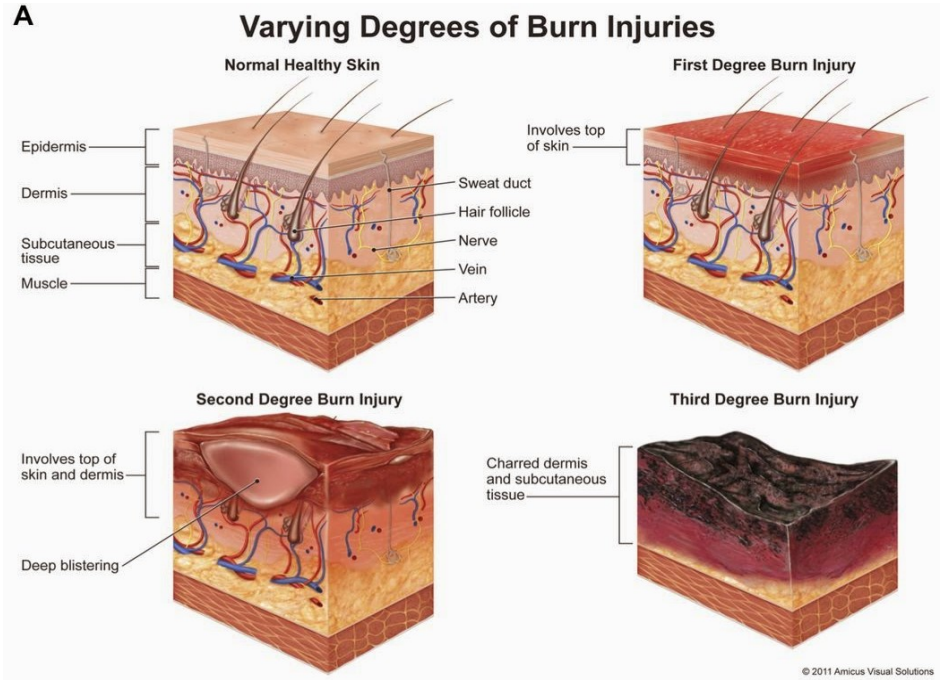


Figure 7: Classification of burns. First-degree burns can heal without treatment. Both second-degree and third-degree burns require excision and grafting because both epidermal basal layer and follicular stem cells have been destroyed.

(Taken from: http://burnnoticeblog.blogspot.com/2015/04/diagnosisclassification-of-burns.html?m=1

1.8 Wound management

Wounds in which the damage is huge, such as in second- or third-degree burns are managed by using skin grafts and wound dressings. The aim of the skin graft is to provide a covering in a rather large wounded area and to accelerate re-epithelisation. Depending on how deep the wound is split thickness and full thickness skin grafts are used. Split thickness graft harvests consist of the epidermis and superficial dermis, with some appendages, whereas in full thickness the whole dermis together with the epidermis and all appendages.

The split thickness skin graft serves as covering for various wound types and promotes epithelialization of the wound and reduces scar formation in the healed wound. Increasing the thickness of the split thickness has been shown to significantly increase the rate of wound healing and wound quality. DeBruler *et al*. (2018) did a comparison study assessing the effect of skin graft thickness on scar development in a porcine burn model. Split thickness autografts of two different thicknesses (660 µm thin) and (1470 µm thick) were harvested from the dorsum of each pig. The skin grafts were engrafted on four full-thickness burns created on four pigs using a custom burn device set at 200º C. Thin autografts were meshed at a ratio of either 1:15 or 1:4 before engraftment and thick

skin was fenestrated with a scalpel. Within 17 weeks wounds covered with thick autografts had little contractions and in those covered with thin autografts, the wounds contracted over time and became rough and raised (DeBruler, et al., 2018).

However, split thickness skin grafts are thin and fragile and are problematic when used over larger surface area, especially near joints where motion can compromise healing. To avoid contractures patient limbs must be immobilised during wound healing (Zeng, et al., 2018). To prevent contraction of large wounds and wounds proximal to joints skin grafts must be stable to withstand shear force caused by moving limbs. For deep and wounds proximal to the joints full thickness skin grafts are used to accelerate healing and to improve the quality of the wound.

To assess the uptake of full thickness skin grafts Struk *et al.,* (2018), conducted a retrospective analysis of full thickness skin grafts that were performed to cover defects below the knee after incisions of non-melanoma and melanoma skin lesions from 2011 to 2016. Full thickness skin grafts were harvested from the contralateral inguinal crease at the deep dermal plane. The grafts were sutured using non-interrupted sutures. Fenestrations were performed and through-and-through tackling sutures were used to secure the grafts to the wound bed and to keep the graft in place a tie-over bolster was used. The lower leg was immobilised by a non-circular above-knee plaster and the patients were instructed to rest on bed for 5 days to avoid venous congestion and declivity (Struk, et al., 2018). Out of 70 patients 64 had over 90% graft take, 3 were moderate (50-90%) and only 3 were lower than 50%. Observations of the wound area after 3 months showed no contractions and scar formation (Struk, et al., 2018). Full thickness skin engrafting has also produced satisfactory results when used to release contractures (Stekelenburg, et al., 2016).

One of the disadvantages of using full thickness skin grafts is the lack or insufficiency of donor sites. Full thickness skin grafts have been previously harvested from the inguinal and abdominal crease (Struk, et al., 2018), thorax, the back, groin, forearm and clavicular region of the chest and the donor site is closed by primary intent (Stekelenburg, et al., 2016; Sivyer, 2018). The same technique of closing the donor site wound has been used in hair transplantation, when skin containing hair is harvested from the occipital region before being divided into slices of follicular units (Coban, et al., 2011). When skin damage is over a large surface area, there won't be any donor sites available. Allografts and xenografts have also been used to cover the wound. However, there are risks involved: the graft can be rejected, or diseases might be transmitted. To solve this major difficulty researchers have developed cultured skin substitutes composed of scaffold materials, growth factors and cells (Zeng, et al., 2018).

1.9 Cultured skin substitutes

An ideal skin substitute should be able to enhance wound healing, decrease pain, be easy to obtain and manufacture, and enable the reconstruction of the epidermis and the dermis. There are various commercial skin substitutes designed for different skin conditions on the market. They are composed of either the extracellular matrix scaffolds, decellularized dermis and cultured cells including keratinocytes and fibroblasts (Limova, 2010).

Cultured keratinocytes obtained from a different sites on the same individual's body can be delivered by spray grafting using fine needle spraying out of a syringe (Gerlach, et al., 2011) or by a Vivostat spray applicator system (Warwick, et al., 2002), or keratinocytes together with fibroblast can be allowed to attach to a collagen-glycosaminoglycan scaffold before delivery. These techniques have been shown to help facilitate wound closure and improve the quality of the wound that require a small amount of donor tissue. However it is important to note that skin derived from these approach lack skin appendages, including important glands and hair follicles (Boyce, et al., 2002; Boyce, et al., 2017).

1.10 Problem identification

Thus, although there has been a lot of progress on improving wound healing and burn tissues, one of the major problems that has not been solved is the regeneration of various components of the skin, including hair follicles, sebaceous glands, sweat glands and other adnexa.

The absence of adnexal structures leads to significant problems regarding the functioning of the skin. Most importantly, the absence of hair follicles and glands over large surface area results in contractures and loss of pliability, which leads to mechanical defects. Another consequence is that the loss of the stem cells in the hair follicle means that the regenerative ability is compromised and could fail. Furthermore, lack of glands results in poor lubrication and flexibility of the skin, further affecting its function.

In order to develop methods for regenerating hair follicles, researchers have been using animal and *in vitro* models to mimic early hair follicle induction. As will be discussed below, progress and success in these endeavours has been somewhat limited justifying ongoing basic research on the subject.

1.11 Hair regeneration studies

As described earlier, hair follicle induction results from the interactions between epithelial and mesenchymal cells in the embryo. The main goal of hair regeneration studies is to mimic these events as best as possible using adult donor stem cells and use them to optimise the interactions between epithelial (keratinocytes) cells and inductive mesenchymal (dermal papilla) cells. The main difficulties are establishing which cells to use, how to grow expand these cell populations which while retaining their stem cells properties, and then and how to bring them together to allow induction to occur.

Early investigators carried out experiments to find out which components are crucial for hair growth and regeneration. Oliver (1966) used techniques of hair follicle amputation described by Cohen (1961) to assess which components are crucial for hair follicle growth and regeneration. In brief, varying lengths of whisker root were cut off from the proximal (lower) end of the vibrissal follicles on the upper lip of the hooded rat. The regenerative capabilities were measured, and it was found that the follicles varied according to length of the cut from the proximal end of the follicle. Follicles in which only the dermal papilla was removed to regrew whiskers of normal length. Follicles in which the hair matrix and the dermal papilla were cut regenerated whiskers 46% of the normal length. The length of the regenerated whiskers kept on decreasing with increasing length of the cut until a point where the follicle didn't regenerate whiskers at all (Oliver, 1966; Ibrahim & Wright, 1982). The results show that the regenerative potential is dependent on the amount of proximal root end of the hair follicle present, where the dermal papilla and the hair matrix are situated. They concluded that there must have been some cellular contamination on follicles that enabled them to regenerate the whiskers without the dermal papilla or the hair matrix or the hair follicles.

To further demonstrate that dermal papilla cells are crucial in hair growth or regeneration, Horne and Jahoda (1986), amputated the hair follicles by removing half of the lower follicle, leaving half or less of the upper region of the follicle in situ. The cut vibrissae shafts were then plucked from the upper segments and the shafts were then available to receive cultured cell implants or to act as nonimplanted controls. The empty follicles either received intact papilla implants, cultured fibroblasts implants or cultured papilla cell implants. Out of 4 follicles that received intact papilla implants 3 grew. Thirty-five (35) follicles that received cultured fibroblasts did not grow. Of the 9 passage 1 papilla cell samples, 6 grew. Passage 2 cells grew in 17 out of 33 implants, passage 3 grew in 4 out of 14 implants and passage 6 did not grow any follicles. These results clearly show that that dermal papilla cells are crucial in hair growth. However, as will be discussed below this feature is lost by subsequent passaging of the dermal papilla cells.

Other researchers have carried out hair induction investigations using dissociated neonatal or embryonic mouse cells. To determine whether freshly dissociated neonatal mouse dermal and epidermal cells can regenerate new hair follicles, Zheng *et al.,* (2005) isolated dermal and epidermal cells from 0-2-day-old GFP-mice from Jackson laboratories (Bar Harbor, Maine), mixed them and directly injected them into the hypodermis of nude mice. Skin patches were harvested and observed under the dissecting microscope then fixed in formalin for haematoxylin and eosin staining and immunohistochemistry. Immunohistochemistry results showed GFP-labelled epithelial cells forming clusters at day 2 of implantation. Ki67 staining showed that epithelial cells were proliferating. Alkaline phosphate staining was positive in the dermal cells, indicating follicular papilla formation. At day 4 the epithelial cells showed early follicle bud-like/peg-like structures. At day 6 the papilla cells are well defined and covered with epithelial cells, forming hair follicle-like structures. At day 12 the harvested

patch appeared as a slightly elevated, grey, round area of skin under the dissecting microscope (Zheng, et al., 2005). This shows that dissociated cells still have “memory’’ (or developmental competence) to regenerate hair follicles when implanted into a permissible environment.

To determine whether the dissociated neonatal mouse epidermal and dermal cells can regenerate hair after *in vitro* propagation, Kageyama *et al.,* (2018) isolated neonatal mouse skin epidermal and dermal cells and cocultured them for 3 days in a non-cell adhesive 96-well plate before transplantation in nude mouse. When observed *in vitro*, the epithelial and mesenchymal cells were able to self-sort and form small hair germs, the mesenchymal cells maintained the expression of dermal papilla signature markers, Alp and versican. Mesenchymal cells also showed enzymatic alkaline phosphate activity and anti-versican antibody staining. The epithelial cells were expressed K15 protein, a marker of hair-follicle stem cells. The hair germs were able to form hair 18 days after transplantation into nude mice (Kageyama, et al., 2018). This shows that neonatal mouse epidermal and dermal cells still maintain their memory even after *in vitro* propagation in a 3-D environment.

Interestingly, there are studies that investigated whether non-hair forming epithelial tissues could be induced by dermal signals to form hair follicles, these studies were aimed at investigating whether other epithelial tissues are only committed to forming only one cell lineage. To determine whether interfollicular epidermis could be induced to from follicular epithelium hair follicle inducing dermal signals, Reynolds and Jahoda., (1992), sandwiched confluent passage 2 or 3 dermal papilla cells obtained from the mid-flank region of a dead, adult hooded rat aged three to twelve months between the split dermal and epidermal components of footpad skin which was cut from the central sole region of a dead, adult hind paw. Before implantation, the reconstituted foot pad skin specimens were incubated overnight at 37º C in rat serum and thereafter implanted on the dorsal skin of adult rat using the silicon chamber method. The implantation site was first incised 10 days prior to create a granulation tissue pocket, that provided vascularisation. The implants were biopsied after 8 weeks, photographed and fixed in formal saline prior to their processing for a standard wax histology. Pelage-type hair fibres were formed in 3 out 6 footpad skin recombinants. However, no sebaceous glands were formed (Reynolds & Jahoda, 1992). The results from this study suggest that adult footpad epidermis could be induced to form hair follicles by hair inducing dermal cells.

Having shown that rat interfollicular epidermis can be induced to form hair follicles by hair inducing dermal cells, Ferraris *et al.,* (1997), aimed to determine whether human epidermal tissue could also be induced to form hair follicles. The question asked in this study is whether human keratinocyte stem cell populations of the interfollicular epidermis are only committed to the production of one cell lineage or whether they can form hair follicles. They took two approaches (1). the recombination of *in vitro* cultured epithelial sheet with dorsal dermis from 19-day rabbit embryo skin (2). stuffing cultured rat

dermal papilla cells in between the epidermis and dermis of human skin which was already engrafted onto nude mice skin.

In the recombination experiments, they made confluent cultures of epidermal sheets by seeding dissociated keratinocytes obtained from healthy female subjects who underwent a facelift cosmetic surgery and the cells were seeded at a density of 1 to 2 x 10^5 cells/cm^2 on a feeder layer of irradiated mouse 3T3 fibroblasts for four weeks. The epidermal sheets were then recombined with dorsal dermis from 19-day rabbit embryo skin and then grafted onto the back of nude mice for 2 to 4 weeks. The samples were first analysed by classical histology. A second set of experiments were repeated so that the samples can be analysed by in situ hybridisation using the Alu probe and by Hoechst staining. In the first series of experiments, one recombinant case developed a few stage 2 hair buds as defined by Hardy (1968) and, hair follicles were at stage 6 or 7 in three cases out of 10 recombinant cases. In the 2nd series of experiments the Alu probe only labelled 5 out of 16 human epidermal implants. In one case the epidermis formed 2 to 4 hair buds associated with rabbit dermal papillae. The epidermis formed stage 6 hair follicles of mixed origin in two cases (Ferraris, et al., 1997).

For wound healing experiments, they engrafted full thickness foreskin of children aged 2 to 10 years and discarded face-lift skin obtained from a healthy 69-year-old woman onto the back of nude mice for two months. Thereafter, passage 2 confluent cultures of rat dermal papilla cells were collected as clumps and transferred into an incision made in the middle of the graft throughout the entire thickness of the engrafted skin. After 3 months ,the engrafted tissue was recovered and analysed by classical histology and immunological staining. Human facelift implants showed two kinds of hair follicles, the pre-existing hair follicles which are of human origin had their keratinocytes and dermal papilla cells labelled by the Alu probe, however, only newly induced hair follicles and not the rat dermal papilla cells were stained by the Alu probe. Foreskin implants formed new hair follicles and sebaceous glands, which were labelled by the Alu probe, indicating that they are of human origin. The results from this study show that epidermal tissue regardless of age and body site can be induced by hair inducing dermal signals to form new hair follicles (Ferraris, et al., 1997).

Having established that epidermal tissue can be induced by dermal tissues to form hair follicles by hair inducing dermal tissue, they went on and investigate whether a different epithelium could also be induced. To do this, Ferraris *et al.,* (2000), recombined rabbit central corneal epithelium with embryonic mouse dermis. They obtained the embryonic hair inducing dermis from the upper lip of 12.5-day and from the back of 14.5-day mouse embryos. To dissociate the dermis from the epidermis they treated the skin tissue with 1.25% trypsin and 2% pancreatin in Ca^{2+} and Mg^{2+}-free Earle's solution for 15 minutes and then stored in DMEM/20% foetal calf serum (FBS) for 10 minutes to 1 hour. Rabbit corneal tissue was carefully excised leaving a 5 mm margin making sure that the limbal tissue did not contaminate the central part of the cornea. The underlying stroma was removed by

treatment with 0.25 EDTA in Ca^{2+} and Mg^{2+}-free Earle's solution. The corneal tissue was then rinsed with Tyrode before recombination with embryonic mouse dermis on agar medium and the recombinants grafted after an hour under the kidney capsule of athymic nude mice for 3, 6, 12 and 21 days. The grafts were assessed by wax histology or embedded in OCT and stored at -80℃ for cryosectioning. The rabbit corneal epithelium which was recombined with embryonic mouse dermis formed a basal layer at day 3, and the newly formed basal layer displayed low K12 expression levels. However, K12 was still predominantly expressed in suprabasal layers. At day 21, K12 staining was in patches in the shedding layers of the epidermis and it was fully replaced by epidermal-type maker, K10 in the suprabasal layers. Simultaneously, at day 6, in both cases involving 12.5-day upper-lip and 14.5-day dorsal mouse dermis recombinants, the rabbit corneal epithelium formed hair bulbs that were associated with condensing dermal cells extending into the dermis. In addition, the new epithelial tissue also had sebaceous glands, granular and cornified layers, resembling native hair forming epidermis (Ferraris, et al., 2000). The results of this study show that an epithelium that is committed to form corneal tissue can transition to form the epidermal and follicular-type epithelium under the influence of embryonic mouse skin dermis signals.

Having shown that adult differentiated corneal epithelium can give rise to hair and interfollicular epidermis, they went on to query the mechanism in which this process transpires. Pearton *et al.,* (2005), followed the same procedure except that the recombinants were recovered at various points between 1 and 30 days and embedded in OCT compound for immunofluorescence or immunochemistry. They also showed that the corneal epithelium loses the expression of corneal-type marker K12 and Pax6, which is a paired homeodomain containing nuclear transcription factor that controls many downstream genes in eye morphogenesis, including K12 expression. Concurrently, the epithelium started expressing K14 and K5 which are normally expressed in the basal layer of the epidermis. Pax6 and K12 expression levels started decreasing at day 2 of recombination and its subcellular localisation shifted primarily to the cytoplasm. This happened concurrently with the formation the basal layer at the dermal-epidermal junction. The basal layer was no longer expressing K12 by day 4, by 6, and Pax6 was only seen in patches. Ki67 staining which indicates proliferative potential of cells was only detected on the second day of recombination concurring with the formation of the basal layer, indicating that the cells in the presumed basal layer have regenerative capabilities. They went on and evaluate how these hair follicles were by looking at the expression of β-catenin, Lef1 and K17 during the recombination process. The lower levels of the epithelium showed an increased β-catenin protein expression as compared to the suprabasal layers on the 2^{nd} day of recombination and by day 4 the staining was restricted to cytoplasm of basal layer cells. By day 9, β-catenin was only found in the basal layer. Concurrently, Lef1 expression was also increasing and it was also detected in some cells in the dermis and by day 7-9 the expression was aligned in clumps in the basal layer. K17, which signals the activation of keratinocytes, appeared as early as day 1,

primarily in the basal layer and by day 2 it was confined in the lower layers. The formed hair pegs at day 7-8 of the recombination expressed high cytoplasmic β-catenin levels and were devoid of Pax6 expression (Pearton, et al., 2005). This study showed that the embryonic mouse dermis can elicit transdifferentiation of differentiated corneal epithelium to form hair follicles and interfollicular epidermis, firstly by switching corneal-type markers and subsequently switching on epidermal type markers and upregulating hair induction signals.

However, these experiments haven't been done in a setting where the cells are passaged for expansion *in vitro*. For clinical purposes inductive mesenchymal and competent epithelial cells will have to expanded *in vitro* to get enough cells to cover large wound areas. For this to happen, mesenchymal cells would have to be cultured in a way that their hair follicle inductive potential is maintained.

There is therefore a need for an effective *in vitro* system to maintain the inductive potential of mesenchymal cells and a system that will promote mesenchymal and epithelial interactions mimicking the *In vivo* hair follicle crosstalk.

1.12 Maintaining hair follicle inductive potential of mesenchymal cells *in vitro*

Considering that hair follicle inductive potential is lost by growing dermal papilla cells in tissue culture dermal papilla cells, investigators have looked at ways to maintain their inductive potential *In vitro*. These experiments took advantage of the fact that embryonic hair follicle formation is initiated by the Wnt signalling and they also tried to mimic the *in vivo* dermal papilla. Inductive dermal papilla cells are characterised by their self-aggregation nature and they have their own signature markers which are distinguishable from the interfollicular dermis. Among others, dermal papilla cells express alkaline phosphatase, versican and the transcription factor Sox2 (Yang, et al., 2012; Xiao, et al., 2017).

1.12.1 Effect of secreted Wnt on inductivity

As discussed above that Wnt/β-catenin signalling is required for the aggregation of mesenchymal cells and follicular differentiation of the interfollicular epithelium during embryogenesis. One way that the inductive potential could be maintained is by taking advantage of the secreted Wnt molecules.

To determine whether the inductive potential of dermal papilla cells in culture could be maintained by the Wnt signalling pathway, Kishimoto *et al.,* (2000) co-cultured dermal papilla cells with Wnt3a producing cells. Lef1, dishevelled and frizzled gene expression levels were increased and when the dermal papilla cells where co-grafted with keratinocytes on the back of nude mice hair growth increased (Kishimoto, et al., 2000).

In addition to co-culturing dermal papilla cells with Wnt producing cells, the medium in which the Wnt producing cells are grown can be harvested and used for induction. The Wnt-conditioned medium was also able to reinstate hair inductivity of dermal papilla cells in culture, even after treatment of

dermal papilla cells with dihydrotestosterone (DHT) (Dong, et al., 2014). DHT impairs hair follicle inductivity by upregulation of DKK1 and through growth inhibition (Kwack, et al., 2008).

In addition to using Wnt producing cells chemicals such as 6-bromoindirubin-3-oxime (BIO) that activate the Wnt/β-catenin signalling pathway could be used to maintain the inductive potential of dermal papilla cells (Soma, et al., 2012). BIO initiates Wnt signalling by inhibiting GSK-3. BIO cultured dermal papilla cells showed increased levels of Wnt signalling molecule Lef1 and dermal papilla specific markers alkaline phosphatase and versican. The BIO treated dermal papilla cells were able to form new hair follicles when combined with murine epidermal cells upon transplantation (Soma, et al., 2012).

These observations demonstrate the regulatory role that Wnt/β-catenin signalling plays for the purpose of hair follicle induction both *in vivo* and *in vitro* as such, Wnt signalling molecules could be used to maintain the hair inductive potential.

1.12.2 Hair follicle induction potential by spheroid cultures of mesenchymal cells

Early passage inductive mesenchymal cells appear to adopt an aggregative growth phenotype and after subsequent passaging they lose this phenotype. This phenotype is correlated with the inductive capabilities of dermal papilla cells (Rushan, et al., 2007).

To reverse the effect of *in vitro* propagation, recent studies have mimicked the *in vivo* dermal papillae by creating a microenvironment that enable dermal papilla cells self-aggregation. Higgins *et al.,* (2010) have shown that subjecting human scalp dermal papilla cells in hanging-drop cultures results in restoration of dermal papilla signature markers. Spheroids were formed within 24 hours of hanging drop culture and they expressed versican protein and also showed a high alkaline phosphatase enzyme activity. In addition to dermal papilla signature markers, conventional PCR for the Wnt signalling molecules, Lef1 and Axin2 showed their gel electrophoresis bands to be thicker than in 2D cultured dermal papilla cells (Higgins, et al., 2010). These observations show that subjecting dermal papilla cells in an environment that allows them to self-aggregate could restore their hair follicle induction potential.

To determine whether the dermal papilla spheroids can form new hair follicles, the dermal papilla spheroids were sandwiched between the separated epidermis and dermis of human foreskin and grafted into SCID mice for 6 weeks. Alkaline phosphatase activity was high in the newly formed hair follicles and the newly formed follicular epithelial comprised of all concentric layers expressing hair-specific markers (Higgins, et al., 2013). However, these experiments were only done for 6 weeks and the hair follicle neogenesis efficiency varied with each biological repeat. Nevertheless, these experiments demonstrated that clusters of mesenchymal cells are able to induce hair follicles in samples of glabrous skin (foreskin). These results open the way for exploring the possibility of using cultured foreskin keratinocytes to induce hair follicles in skin transplants. This will be discussed below.

1.13 Can cultured keratinocytes respond to dermal signals?

Adult tissue stem cell populations either in the basal layer of the epidermis or in the bulge region of the hair follicle are responsible for skin homeostasis and hair follicle cycling, respectively. The stem cells in the basal layer of the adult epidermis respond to dermal signals and replenish the layers of the epidermis. During injury dermal signals instruct the epithelial cells to migrate and cover up the damaged site. Bulge stem cells receive signals form dermal papilla cells during the anagen stage specifically to form hair and they can also be recruited to the epidermis in an event of an injury (Levy, et al., 2005). However, these processes cannot be achieved when the damage is severe, cells will need to be expanded *in vitro* and this expansion . mustn't compromise their ability to respond to dermal signals (i.e. they must retain competency).

To maximise keratinocytes needed for hair follicle reconstitution Zhang *et al*., (2012), used organ culture method and a two-step trypsinisation procedure to harvest high quality hair follicle stem cells that can be expanded *in vitro* without losing their competency. Intact adult rat vibrissae hair follicles obtained by microdissection were each cultured in a well of 24-well plate or 10 in every 60-mm dish in William's E growth medium. The hair follicles adhered to the plate within 3-7 days and colonies grew from the bulge area. To purify the hair follicle stem cells from the growing fibroblasts, a two-step trypsinisation procedure was used. Taking advantage that hair follicle colonies adhered to the plate tightly, the culture was first treated with 0.1% trypsin- 0.008% EDTA at room temperature for 3-5 minutes for fibroblasts to detach from the surface. To lift the hair follicle stem cells, the cells were first rinsed with PBS, followed by inactivation of trypsin by FBS and thereafter treated with trypsin/EDTA for 5 minutes at 37º C. The passaged hair follicle stem cells were then co-cultured with fibroblasts for further expansion. The hair follicle stem cells formed large colonies within 2 weeks after being plated, and furthermore, they were also able to achieve a logarithmic growth following a 15-days growth curve. To determine whether the colonies are competent, quantitative RT-PCR analyses revealed that passage 7 and 12 hair follicle stem cells expressed high levels epithelial basal layer markers, K14 and alpha-6 integrin, the epithelial stem cell marker p63, and bulge stem cell marker, K15 when comparing them to fibroblasts. To investigate whether the cultured hair follicle stem cells can reconstitute hair follicles, passage 8 follicle stem cells infected with lentivirus containing GFP construct were mixed with neonatal dermal fibroblasts and transplanted to the back of nude mice using silicon chambers. The transplanted cells were able to form new hair follicles, epidermis and sebaceous glands (Zhang, et al., 2012). While this important result reveals that epithelial cells (from rat) can be cultured and induced , the process is very laborious and difficult and very time consuming to acquire sufficient intact hair follicles and would therefore not be likely to be practical for clinical use.

In a similar way, Wang *et al*., (2016), investigated whether mouse epidermal stem cells are capable of regenerating epidermal appendages when combined with neonatal mouse dermal cells. Epidermal stem cells were obtained from postnatal day 3 mice were cultured in CnT-07 progenitor cell-targeted

(PCT) epidermal keratinocyte medium (CellnTec Advanced Cell Systems, North Carolina, USA) and purified by their ability to rapidly attach to the surface of plastic culture dishes (Strachan, et al., 2008). The rapidly attached cells expressed stem cell markers CD49e (alpha-6 integrin) and K15. However, CD49e expression declined with increase in passage number of the cells. To examine whether the epidermal stem cells could be induced to form hair, they were mixed and encapsulated in Matrigel™ with neonatal mouse dermal cells and transplanted on the back of nude mice for 3 weeks. The number of hair follicles formed were directly proportional to the passage number of the epidermal stem cells (Wang, et al., 2016). These results suggest that multipotency is crucial in the ability of keratinocytes to from new hair follicles.

To get an idea which source of keratinocytes can be efficiently used for regeneration, a study performed by Kobayashi *et al*., (2009), compared the characteristics of canine interfollicular and follicular keratinocytes. They used colony-forming assays to compare their proliferative capacity of each of these keratinocyte populations. Equal numbers of interfollicular and follicular keratinocytes (2500/dish) were seeded onto 60-mm dishes and cultured for 2 weeks. The colonies were fixed with 4% paraformaldehyde and stained with a 1:1 mixture of Rhodamine and Nile blue solution. The number of colonies formed from follicular keratinocytes was 5.8-fold higher than that of interfollicular keratinocytes. In addition, the follicular keratinocytes colonies also had larger diameter (Kobayashi, et al., 2009). The results suggest that follicular keratinocytes have a high regenerative capacity as seen by their ability to form larger number of large sized colonies, the only setback to their use being that it's difficult to acquire them.

In addition to selecting epidermal cells that could be efficiently expanded, researchers have also explored whether keratinocytes that have previously been co-cultured with mesenchymal cells can be more efficiently expanded. To determine the effect of mesenchymal cells on keratinocytes, Limat *et al.,* (1993), co-cultured outer root sheath keratinocytes obtained from plucked hair follicles from the occipital region of the scalp with human mesenchymal cells in a Transwell system consisting of microporous inserts, pore size 0.45 µm. Outer root sheath keratinocytes were plated in KGM inside the insert and mesenchymal cells were either cultured at the underside of the insert membrane or at the dish bottom. The co-cultured outer root sheath keratinocytes rapidly attached to the surface and formed more keratinocyte colonies, as compared to monocultures. In addition, they tested whether an increase in the number of mesenchymal cells would have an effect on keratinocyte cell growth. Cell growth was assessed by ^{3}H-thymidine incorporation assay, which measures cell proliferation by directly measuring DNA synthesis. They found that outer root sheath keratinocyte growth was increased with an increase in the number of mesenchymal cells (Limat, et al., 1993). The results indicate that mesenchymal cells are crucial for keratinocyte adhesion to the tissue culture plate surface and growth, with dosage of soluble factors playing an important role.

Consistent with the above discussion, the effect of increasing the number of mesenchymal cells was investigated on their ability to effect regeneration of the epidermis. Increasing numbers of human fibroblasts were plated on collagen matrices and after overnight incubation, keratinocytes obtained from human mammary skin surgery were seeded and subsequently cultured for 2 weeks at the air liquid-interface. The regenerative capacity was evaluated by assessing the proliferation index (PI), which is the ratio of Mb 67-positive nuclei to the total number of basal cells (x100). MB67 (Ki-67) is a nuclear protein expressed in proliferating cells. The results were compared to that of human skin. The quality of tissue architecture was increased with increase in the number of fibroblasts plated as observed in haematoxylin and eosin stained tissues The PI was increased with increasing cell number (El-Ghalbzouri, et al., 2002), indicating that more fibroblasts providing extra factors are needed for efficient epidermal regeneration.

The next matter to evaluate whether mesenchymal cells can maintain keratinocytes over a long-term. To determine whether mesenchymal cells can support proliferation and colony formation following subsequent keratinocyte passaging, Chan *et al.,* (2015), cultured outer root sheath keratinocytes atop lethally irradiated 3T3J2 fibroblasts. Both passage 2 and 87 showed a higher proliferation activity and a uniform cell morphology, indicating that the high passage keratinocytes did not lose their stem cell phenotype (Chan, et al., 2015). However, the 3T3J2 co-cultured keratinocytes didn't form new hair follicles when combined with dermal papilla and transplanted to nude mouse. To determine whether the dermal papilla co-cultured keratinocytes were primed to form new hair follicles they first did qRT-PCR for β-catenin to verify whether the Wnt/ β-catenin signalling pathway (which is associated hair follicle initiation) is active. β-catenin gene expression levels increased for each day spent in the co-culture. The co-cultured passage 36 keratinocytes readily formed new hair follicles when combined with passage 3 dermal papilla cells (Chan, et al., 2015). The results suggest fibroblasts can support high passage keratinocyte cell growth, but not as well as dermal papilla cells.

As shown above that there seems be differences on the effect of mesenchymal cell types on keratinocytes, Hill *et al.,* (2013), investigated the difference between mesenchymal cell types on their ability to elicit proliferation. The keratinocytes formed more colonies when co-cultured with dermal sheath and dermal papilla cells as compared to dermal fibroblasts (Hill, et al., 2013), indicating that dermal sheath and dermal papilla cells provide better regenerative capacity, this could be because they natively provide support to highly mitotic hair matrix epidermal cells.

In addition, there seems to be differences also in the passage number of mesenchymal cells. To determine the role which passage number plays, Reynolds *et al.,* (1991), co-cultured rat keratinocytes with low and high passage dermal papilla cells and dermal fibroblasts. Keratinocytes co-cultured with low passage dermal papilla cells and fibroblasts rapidly attached and showed a higher proliferative capacity. However, high passage dermal papilla cells still showed a higher attachment and

proliferative capacity as compared to low passage fibroblasts (Reynolds, et al., 1991). The observations indicate that dermal papilla cells naturally provide a better keratinocyte regenerative capacity as compared to dermal fibroblasts.

1.14 Use of immortalised keratinocytes

It has been shown that primary human keratinocytes undergo only about 15 to 20 population doublings *in vitro* and once they reach quiescence they don't respond to exogenous mitogenic stimuli (Chio, et al., 2017) . This somewhat limits their usability in experimental approaches and when many cells are needed, the spontaneously immortalised HaCaT cells and other immortalised human keratinocyte cell lines have been used to overcome this problem. HaCaT cells retain a remarkable capacity for normal differentiation by being capable of expressing differentiation-type keratins, K1 and K10 even after multiple passages. In addition, HaCaT cells do not need special medium conditions to grow, as they can be cultured in DMEM plus 10% FBS (Boukamp, et al., 1988).

To evaluate the differences between the normal human keratinocytes and HaCaT cells Micallef *et al* (2008) compared the differentiation state of normal human keratinocytes and HaCaT cells when cultured in KSFM containing high calcium concentration (1.2 mM), as it has been reported that high calcium induce growth arrest and differentiation of cultured keratinocytes. All cells were seeded at a density of 3 x 10^4 cells/cm^2 to allow the cells to adhere to the surface for 24 hours in low calcium KSFM (0.9 mM). The medium was then changed to high calcium KSFM. The MTT colorimetric assay was performed to measure cell proliferation and RNA for RT-PCR was extracted at 1,3, 6, 9 and 13 days. The proliferation rate in both high and low calcium KSFM was the same at day 1 in normal human keratinocyte cultures and thereafter slowed down in high calcium KSFM. In contrast, the proliferation rate showed no difference in the first 3 days in both high and low calcium cultured HaCaT cells. Thereafter, the proliferation rate increased from day 6 to 13; surprisingly, high calcium cultured HaCaT cells had a higher proliferation rate. Phase contrast images showed that cells had morphological changes associated with differentiating keratinocytes. The cells became small and cobblestoned specifically in normal human keratinocytes, whereas those in low calcium KSFM were flat and spread out. RT-PCR analyses for differentiation-type markers showed that K1 and K10 expression were detected at day 1 in high calcium KSFM and at day 3 for low calcium KSFM in normal human keratinocytes. In contrast, K1 and K10 were not detected at day 1 for both high and low calcium KSFM in HaCaT cell cultures. They were detected at day 3 in high calcium KSFM and at day 6 for low calcium KSFM (Micallef, et al., 2008). The results suggest that HaCaT cells maintain their undifferentiated state longer than normal human keratinocytes. This could be the reason why they can be used at later passages.

Early investigators carried out experiments on whether HaCaT cells are capable of forming a normal epidermis. Boukamp *et al.,* (1988) transplanted HaCaT cells onto nude mice either as cell

suspensions or as intact cultures growing on glutaraldehyde-fixed collagen gels. For cell suspensions, they first inserted a hat-like silicon chamber by an incision of the back of nude mice skin and kept it in place by tightly fixing the wound margins of the skin with wound clips. The HaCaT cell suspension was injected through the chamber onto the muscle fascia at the interscapular region of 6-week old nude mice. The transplants were excised at weekly intervals for histological analysis.

Prior to generating intact HaCaT cell cultures, they gelled 300 µL medium containing 3 mg/ml collagen type I from mouse tail tendon in silicon culture chambers (Renner GmbH) by exposure to 1.9% ammonia vapour for 1 hour and fixed with 45 glutaraldehyde for 1 hour. Thereafter, they seeded 2 x 10^5 HaCaT cells in 200 µL 4x MEM, 5% FCS medium and placed the chambers in Stanzen petri-dishes (Greiner uS, Nurtiugen, FRG) to allow medium access on the below and incubated at 37º C in a humidified gassed atmosphere for 24 hours. They covered the chamber with a hat-like transplantation chamber and transplanted as a whole unit onto the muscle fascia of nude mice.

The injected HaCaT cell suspensions formed large encapsulated cysts that contained stratified keratinising epithelium and after 1-2 weeks of transplantation the cysts epithelium developed a regular tissue architecture including stratum granulosum and corneum. In contrast, intact HaCaT cells cultures formed the epithelium after a delay of 1-2 weeks. However, the reconstituted tissue was almost perfect and resembled the native skin epidermis. This was because the HaCaT cells growing on glutaraldehyde-fixed collagen gels had no contact to host tissue which prevented encapsulation (Boukamp, et al., 1988). The results indicate that HaCaT cells are capable of reconstituting a multi-layered epithelium and they can form a well-structured epithelium if transplanted with proper scaffolding.

To determine whether the transplanted HaCaT cells are able to form a differentiated epithelium with most characteristics of human epidermis, Breitkrentz et al., (1998) seeded passage 35 to 40 HaCaT cells on type 1 collagen gels alone or either with passage 5 to 7 human fibroblasts embedded in gels and incubated them submerged in medium for 1 day before transplantation. The cocultures were transplanted onto nude mice using a transplantation chamber. The transplants were dissected at different time points for haematoxylin and eosin staining and for immunofluorescence. To determine the proliferation activity of HaCaT cells nude mice were injected with 100 µL 5-bromodeoxyuridine (BrdU) and deoxycytidine (both Sigma, St Louis/USA) in PBS two hours before dissection.

The HaCaT cells formed an undifferentiated multi-layered epithelium at day 4 and at day 7 the basal layer and flattened suprabasal layers were observed. Keratinisation became apparent at day 14. Complementary to the formed epithelia, BrdU labelling was observed in all cells of the multi-layered epithelium at day 4. After a week the BrdU was restricted to the basal layer of the epithelium. Immunofluorescence analyses showed staining of differentiation markers, K1 and K10 on suprabasal layers at day 4 and basal cell layer marker, K14 was observed mainly in the basal layer between one

to weeks (Breitkreutz, et al., 1998). The results indicate that HaCaT cells are capable of forming an epithelium that is naturally akin to the native epidermis as shown by the formation multi-layered epithelium that expressed both basal layer and suprabasal layers markers.

Having shown that HaCaT cells can form an epithelium that resembles the native epidermis, they went on and evaluate whether HaCaT cells can form a normally structured epithelium *in vitro*. They seeded 1.3-3.8 x 10^5 per cm^2 HaCaT cells on top of human de-epidermised dermis on filter inserts with or without pre-cultivated human dermal fibroblasts. The co-cultures were harvested at week 1, 2 and 3 for histological analysis. To determine the proliferation rate BrdU and deoxycytidine were added to the culture medium two hours before fixation.

The quality of the reconstructed epidermis was depended on the number of pre-cultivated fibroblasts on the co-cultures. Co-cultures with 5 x 10^5 fibroblasts formed a well-structured multi-layered epithelium as compared to 2 x 10^5 and 1 x 10^5 at day 7. The basal layer with cuboidal cells was observed at week 2 and the epithelium keratinised at week 3. In contrast, fibroblast free cocultures formed a poorly organised epithelium at day 18. HaCaT cells in the basal layer and suprabasal layers were all labelled by BrdU at week 1. BrdU labelling was restricted to the basal layer at week 2 and 3. K1 and K10 were all strongly expressed in expressed in the suprabasal layer at day 21 (Schoop, et al., 1999). The results indicate that HaCaT cells are capable of epidermal differentiation *in vitro* as shown by the formation of multi-layered epithelium and the switch from undifferentiated epithelium to one that expressed suprabasal layer markers.

1.15 Research question and approach

All these hair follicle reconstitution assays support the principle that epithelial and mesenchymal components are critical in induction of hair follicle

Although hair induction has been achieved in animal models, the ability to induce hair follicles using human skin keratinocytes and human mesenchyme cells has not been achieved. The long-term aim of the project (in Kidson Lab) is to develop a model of hair follicle induction using human mesenchymal cells and adult keratinocytes (Alice Brown, MSc). This would lead the way towards a clinically relevant model. The broad aim towards this end was to determine/establish the conditions under which human foreskin keratinocytes would be induced by dermal signals.

1.16 Specific Objectives

The specific objectives of this research study are:

1) To determine whether neonatal mouse mesenchymal cells express dermal papilla signature genes.
2) To evaluate the effect of cell-to-cell contact on neonatal mouse mesenchymal cells to assess whether dermal papilla signature genes are maintained.

3) To determine the differentiation state of human foreskin keratinocytes and to assess their proliferative and inductive potential.
4) To evaluate the effect of neonatal mouse mesenchymal cells on human foreskin keratinocytes in co-culture by assessing their ability to elicit early hair induction signals.
5) To evaluate the effect of Wnt conditioned medium on human foreskin keratinocytes by assessing their ability to elicit early hair induction signals.

For the purpose of this dissertation the following markers were used to measure the inductive potential of neonatal mouse mesenchymal cells:

1.17 Versican

Versican is a chondroitin sulphate proteoglycan that is mainly found in the extracellular matrix. It plays a role in adhesion, migration, proliferation and differentiation (Yang, et al., 2012). It highly expressed in the dermal papilla as compared to the connective tissue sheath and other skin fibroblasts (Elliott, et al., 2016). Following subsequent cell passaging dermal papilla cells lose versican expression (Osada, et al., 2007). As predicted, when mesenchymal cells are clustered versican gene expression is upregulated or maintained. This technique mimic *in vivo* dermal papilla (Miao, et al., 2014).To show that versican is crucial in the aggregative nature of dermal papilla cells Feng *et al*. (2010) used small interfering RNA to ablate versican expression. Subsequently after ablation dermal papilla cells were no longer aggregating *in vitro* (Feng, et al., 2010).

1.18 Alkaline phosphatase

Alkaline phosphatase is a hydrolase that releases phosphate groups in a mild alkaline environment and its expression on dermal papilla cells is related to trichogenicity (Yamauchi & Kurosaka, 2009; Soma, et al., 2012; Higgins, et al., 2013). Alkaline phosphatase expressing dermal papilla cells are characterized by their aggregative growth nature *In vitro* (Pearton, et al., 2005). Alkaline phosphatase expressing cells dermal papilla cells have hair induction capabilities (Driskell, et al., 2009).

1.19 Sox2

Sox2 is an SRY-related transcription factor encoding a high mobility group (HMG) DNA binding motif (Lang, et al., 2011). It is involved in the neurogenesis and it represents undifferentiated cellular population (Lang, et al., 2011; Driskell, et al., 2012; Ahfeld, et al., 2017). Sox2 has been reported to be involved in hair follicle development. Sox2 is strongly expressed in dermal papilla cells of the 1st and 2nd waves, which are guard and aw/auchene hairs respectively and not on 3rd wave hair follicles which are zigzag hairs (Driskell, et al., 2009; Clavel, et al., 2012). Sox2 positive dermal papilla cells have been shown to regenerate hair follicles when implanted in mice, and just like versican and alkaline phosphatase, Sox2 gene expression is lost *In vitro* (Driskell, et al., 2012)

2 Chapter 2: Materials and Methods

2.1 Tissue Culture

To ensure a sterile environment at all times for tissue culture, all surfaces in the Bio-Flow Safety Cabinet Class II and consumables or reagents introduced into the hood were wiped with 70% ethanol.

2.1.1 Isolation of primary cells

2.1.1.1 Isolation of neonatal mouse mesenchymal cells

Neonatal mouse mesenchymal cells were isolated from embryonic day 18.5 (4 mice), embryonic day 19.5 (6 mice), post-natal day 0 (5 mice) and post-natal day 2 (4-5 mice in 3 different occasions) mice killed by decapitation using scissors and were obtained from the University of Cape Town UCT Faculty of Health Sciences, Research Animal Facility.

All surfaces, equipment and the container carrying carcasses were sterilised, first with 10% bleach and then 70% ethanol before dissection.

Carcasses were wiped with 70% isopropanol cleansing swabs and rinsed in phosphate buffered saline (PBS) [Sigma-Aldrich, USA] supplemented with antibiotics (100 U/ml penicillin /100µg/ml streptomycin) (Sigma-Aldrich, USA).

Dorsal skin was incised by lifting the skin with blunt forceps and cutting a small piece of skin off with a pair of scissors. The incised skin tissue was further reduced to 10 mm X 10 mm pieces using scalpel blades then submerged in 5mg/ml of dispase solution overnight at 4℃. The dispase was removed and 0.1% Trypsin/0.05% EDTA added to the skin tissue and incubated at 37º C for 1 hour. The epidermis was peeled off using a pair of forceps and discarded.

The dermis was transferred to a 10-cm petri dish, where it was minced into a sludge using scalpel blades. The sludge was transferred to a 3.5-cm tissue culture plate filled with 0.4% (w/v) collagenase and further dissociation was achieved by trituration through a 1.1 x 38 mm syringe needle. This finely minced sludge was incubated at 37º C for 1 hour and then transferred to a 50 mL tube filled with PBS supplemented with antibiotics and centrifuged for 5 minutes at 1000 g twice to further dilute the collagenase.

The supernatant was discarded and the tube transferred to the Bio-Flow Biological Cabinet Class II where the pellet (consisting mostly of single cells or small cell clumps) was resuspended in Dulbecco's Modified Eagle Medium (DMEM) [Highveld Biological, South Africa] supplemented with 10% foetal bovine serum (FBS) Gibco, USA] and antibiotics. The suspension was manipulated to ensure even dispersal of the cells on the base of the dish and thereafter cultured in a water-jacketed 5% CO_2, 95% atmosphere and 65% humidity incubator at 37º C.

2.1.1.2 Isolation of human foreskin cells

Neonatal foreskin tissue was obtained from surgeries in Cape Town as discarded tissue after circumcision. One foreskin tissue was collected in 10 mL DMEM supplemented with antibiotics and stored at 4º C. The foreskin tissue was washed with PBS supplemented with antibiotics, cut into smaller pieces (10 mm X 10 mm) and placed in a 3.5-cm tissue culture plate filled with 5 mg/ml dispase and incubated overnight at 4º C. Using a pair of forceps, the enzymatically treated skin was transferred to a drop of PBS containing antibiotics on a petri dish and the epidermis was stripped off using a pair of forceps.

2.1.1.2.1 Keratinocyte Isolation

To isolate keratinocytes from the epidermis, epidermal sheets were transferred into a drop of Trypsin/EDTA on a petri dish lid minced into the smallest possible pieces using scalpel blades and transferred to a 50 mL tube filled with 5 mL Trypsin/EDTA. The tube was incubated in a water bath for 15 minutes at 37º C with rocking at 5-minute intervals until the trypsin supernatant became cloudy. The dissociated tissues/cells were then triturated with a pre-wet blue tip until all skin pieces were broken up (about 20 times). 5 mL DMEM supplemented with 10% FBS was then added to inactivate the trypsin and the cells pelleted by spinning at 1000g for 10 minutes. The supernatant was discarded, and the pellet resuspended in 1 mL keratinocyte serum-free medium containing 50 mg/L bovine pituitary extract and 5 µg/L human recombinant epidermal growth factor (KSFM) [Gibco, USA] and cells seeded in tissue culture plates

2.1.1.2.2 Fibroblast Isolation

To isolate skin dermal fibroblasts, the dermis was placed under a cover slip on a 3.5-cm tissue culture plate in DMEM supplemented with 10% FBS and antibiotics and the cells were allowed to emerge from the tissue for a period of 1-2 weeks until confluent patches of cells could be seen around the tissue. The coverslip was then transferred to a separate dish and the cell culture continued.

2.1.2 Sub-culturing cells

In order to expand on the stock of primary cells, successive passaging in increasing dish sizes was performed. Cells from a 6-cm tissue culture plate were passaged to two 6-cm tissue culture plates or to one 10-cm tissue culture and from 10-cm tissue culture plate to two 10-cm tissue culture plates. In brief, the medium was removed, and the cells washed with sterile PBS followed by incubation in Trypsin/EDTA at 37º C for 5 minutes. Once the cells had rounded and detached from the dish (this was monitored under the microscope) the Trypsin/EDTA action was neutralised by adding DMEM supplemented with 10% FBS. The remaining cells were washed off the dish with additional medium and transferred to a 15 mL tube to be pelleted by centrifugation at 1000g for 3 minutes. The supernatant was aspirated, and the cells resuspended in 1 mL of DMEM supplemented with 10% FBS and antibiotics.

2.1.3 Freezing cells

In order to preserve cells long term for future experiments, cells were cryopreserved in DMSO (Sigma) -containing medium in liquid nitrogen tanks. In brief, cells from a confluent 10-cm tissue culture plate, were enzymatically lifted with Trypsin/EDTA and pelleted by centrifugation. The pellets were resuspended in ice cold freezing medium (DMEM or KSFM supplemented with 20% FBS and 10% DMSO) and 1 mL aliquots were pipetted on ice into 2-to-3 2-ml cryovials (Sigma). These were transferred to -80º C in a precooled storage box padded with cotton wool to ensure slow cooling at a rate of -1º C/min. The vials were transferred to liquid nitrogen tanks after 2-7 days.

2.1.4 Thawing

DMSO is toxic to cells at high temperatures and therefore, frozen cells were thawed quickly at 37º C until a small ice block remains in the vial. The vial was thoroughly but quickly wiped with 70% alcohol and transferred to the hood where the thawed cell suspension was transferred to a 15 mL tube containing pre-warmed DMEM supplemented with 10% FBS. The cells were pelleted by centrifugation at 1000g for 3 minutes and resuspended in 1 mL DMEM supplemented 10% FBS and antibiotics or in KSFM for keratinocytes.

2.1.5 Spheroid culture-Hanging drop

In order to mimic the dermal papilla, neonatal mouse cells were cultured in a 3D environment as previously described (Topouzi, et al., 2017). Neonatal mouse mesenchymal cell pellets were resuspended in DMEM supplemented with 10% FBS and antibiotics and the cells counted on a Haemocytometer. The medium was adjusted to a cell concentration of 300 cells/µL and the cell suspension mixed repeatedly while depositing 10 µL drops onto the inverted lid of a tissue culture plate. The inverted lid containing the cell drops was gently inverted onto its base with each drop hanging from the lid. The hanging drops were incubated at 37º C for 48 hours to promote 3D spheroid growth.

2.1.6 Preparation of Fibroblast-Conditioned medium for keratinocyte culture

Culturing keratinocytes with fibroblast-conditioned medium increases cell attachment efficiency and minimises cell loss during seeding (Chowdhury, et al., 2012). To prepare fibroblast-conditioned medium fibroblasts were grown to 80-90% confluency in DMEM supplemented with 10% FBS. The medium was removed, and the cells washed twice with PBS to remove excess FBS. DMEM/F12 (1:1) containing only antibiotics was added to the plate and the conditioned medium was harvested in two-day intervals for a total period of 4 days. In order to remove floating fibroblasts that could contaminate subsequent cultures, the medium was transferred from the plate to a 15 mL tube, centrifuged at 3000g for 10 minutes and syringe-filtered through 0.2 µm filters before storing at -20º C.

2.1.7 Neonatal mouse mesenchymal cells and human foreskin keratinocyte co-culture

To determine the effect of neonatal mesenchymal cell-derived diffusible factors on keratinocyte growth. Keratinocytes were cultured on a Geltrex® LDEV-Free Reduced Growth factor basement

membrane Matrix (Gibco) coated 6-well cluster plate and allowed to attach for two days. Simultaneously, neonatal mouse mesenchymal cells were cultured in 12-mm diameter inserts (0.4 µm pore size) and transferred to the keratinocyte cluster well on the second day. The co-culture was maintained in serum free KSFM:DMEM (1:1) for 3 days and the formation of colonies were monitored. To count the number of colonies generated, 0.5 cm^2 gridlines with assigned numbers were drawn on the base of the cluster well and keratinocyte colonies (colonies with > 3 keratinocytes) were manually counted in each block of the grid as they appeared.

2.2 Gene expression analyses in mesenchymal cell/ Keratinocyte co-cultures

2.2.1 RNA extraction

The Tripure Isolation Reagent (Roche, USA) was used and the protocol was followed as specified by the manufacturer. In brief, 0.5-1 mL Tripure reagent was added to the tissue culture plate and the cells lysed by trituration. The lysate was transferred to an Eppendorf tube and either stored at -80º C for up to 1 month or processed immediately. Briefly, the tube was incubated 5 minutes at room temperature, chloroform (0.2 mL per 1 mL Tripure Isolation Reagent) was added and the tube mixed vigorously for 15 seconds. The phases were allowed to separate by standing 10 minutes at room temperature. The phases were further separated by centrifugation at 12 000g for 15 minutes at 4º C. The upper aqueous phase transferred into a new Eppendorf tube. Isopropanol (0.5 mL per 1 mL Tripure Isolation Reagent) was added and the tube mixed by inversion and subjected to overnight incubation at -80º C. The tubes were centrifuged at 12 000g for 10 minutes at 4º C to pellet the precipitate containing RNA/salt complexes. The supernatant was removed, and the pellet washed with twice with 75% ethanol to remove salts and centrifugation at 7 500g for 5 minutes at 4º C (pellet was dislodged by a vortex mixer before spinning). The pellet was then airdried at room temperature for 10 minutes and dissolved in 20 µL SABAX injection water. The RNA solution was incubated for 10 minutes at 55º C in order to promote the resolubilisation of the RNA.

The concentration of RNA was quantified by measuring OD at 260 nm using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). RNA integrity was determined by agarose gel electrophoresis. Briefly, 1 µg of RNA was mixed with water and a 1x Orange Loading Dye (Thermo Scientific) and loaded on a 1% (w/v) agarose gel (Sigma-Aldrich) containing 1 mg/ml ethidium bromide (Promega, USA). An electric current was applied at 80 volts for 20 minutes.

RNA bands, 28s, 18s and 5s, were visualised by using a UV light Transilluminator (Uvitec) and captured using the Canon EOS 1200D camera.

2.2.2 cDNA synthesis

For the conversion of RNA into cDNA, 1 µg single stranded RNA was incubated with 61 µM Oligo-dT for 5 minutes at 70º C and then placed on slushed ice for 5 minutes. The RNA was reverse transcribed in a 20 µL reaction mixture containing dNTP (1 mM), MgCl2 (2.5 mM), 1X RT buffer, 20U/µL RNase

inhibitor (Promega, UK). and 1 µL 1U/µL MMLV-RT (Promega, UK). The reaction mixture was incubated at 42º C for 1 hour and thereafter stored at -20º C. Each run contained a negative control, with Sabax water (Adcock Ingram, South Africa) instead of RNA, as well as a control for genomic DNA contamination with Sabax water replacing the reverse transcriptase.

2.2.3 Quantitative Real-Time PCR (qPCR)

Gene expression was done by using the StepOne Plus RT-PCR system (Applied Biosystems, USA) as recommended by the manufacturer. The reaction mixture consisted of 5 µL PowerUp SYBR Green Master Mix (Applied Biosystems), 2.6 µL SABAX water, 0.4 µL 10 µM of forward and reverse primers and 2 µL cDNA. The reaction conditions comprised a denaturation step of were 94º C for 5 minutes followed by 40 cycles of amplification comprising denaturation at 94º C for 10 seconds, annealing at 60º C for 30 seconds and extension at 72º C for 30 seconds. The relative gene expression was normalised to mGusb and β-Actin, for mouse and human cells, respectively. Quantification was performed using the comparative Ct ($2^{ddC}t$) method.

2.2.4 Primer design

Mouse primers used in this study were designed using the Nucleotide Database (https://www.**ncbi**.nlm.nih.gov/) site and the gene sequence alignment confirmed by Nucleotide Blast tool (https://www.**ncbi**.nlm.nih.gov/**BLAST**/). Primer oligo sequence properties were further analysed by IDT Technologies OligoAnalyzer Tool (https://www.**idt**dna.com/calc/**analyzer**).

Primers were designed using the following guidelines:

1. Product size was set between 110 and 300 base pairs and those that make the smallest product were selected.
2. Primer sets were designed to lie on two exons to eliminate amplifying genomic DNA.
3. The GC content was set between 40 and 60%
4. The melting temperature (TM) was set between 55º C and 65º C.
5. Where possible, primers whose 3' end bases contained GG, G, CC, C or CG were selected, to avoid specific binding.
6. Primer length was designed to be between 17-22 bp.
7. Primers with runs of 5 bases were avoided.

Mouse Primers were synthesized by the UCT Molecular & Cell Biology (MCB) Oligo Synthesis Service. Human primers were designed by a previous MSc student, Alice Brown, and were synthesised by IDT (IDT, USA) and reconstituted according to the instructions of Whitehead Scientific (Whitehead Scientific, South Africa).

The synthesised primers were tested using cDNA derived from mouse embryonic stem cells . Conventional PCR was used to qualitatively detect gene expression or amplification of Alp, Sox2 and

Vcan. PCR products were visualised on a 2% agarose gel, stained with ethidium bromide and run for 1 hour at 100 volts. The bands were visualised using the UV light Transilluminator (Uvitec) and captured using the Canon EOS 1200D camera (Fig. 8).

Table 2: Mouse RT-PCR and quantitative RT-PCR Primer sequences

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product size (bp)
Mouse Glucuronidase (mGusb)	ACTGACACCTCCATGTATCCCAAG	CAGTAGGTCACCAGCCCGATG	
Alkaline phosphatase (Alp)	TCCGTGGGCATTGTGAC	GCCATCTAGCCTTGTACC	267
Sox2	TAAGTACACGCTTCCCGGAG	AGCCGTTCATGTAGGTCTG	289
Versican (Vcan)	ACGTCCCCTGCA ATTACC	TTAGGCATTGCCCATCTCC	212

Table 3: Human RT-PCR and quantitative RT-PCR Primer sequences

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product size (bp)
β-Actin	GCAAAGACCTGTACGCCAAC	ACATCTGCTGGAAGGTGGAC	
β-Catenin	AAAATGGCAGTGCGTTTAG	TTTGAAGGCAGTCTGTCGTA	100
Nuclear factor-kappa-B (NF-ϰB)	CCTGGATGACTCTTGGGAAA	CTAGCCAGCTGTTTCATGTC	75
Keratin 10	TTGGTGGAGGTAGCTTTCGTGGAA	AGAAGGCCATCTCCTCCAAAT	80
Keratin 5	ACAAGGTTCTGGACACCAAGTGGA	AGGTCCTGCATGTTTCTCAGCTCT	100
Lef1	CCAGCTATTGTAACACCTCA	TTCAGATGTAGGCAGCTGTC	

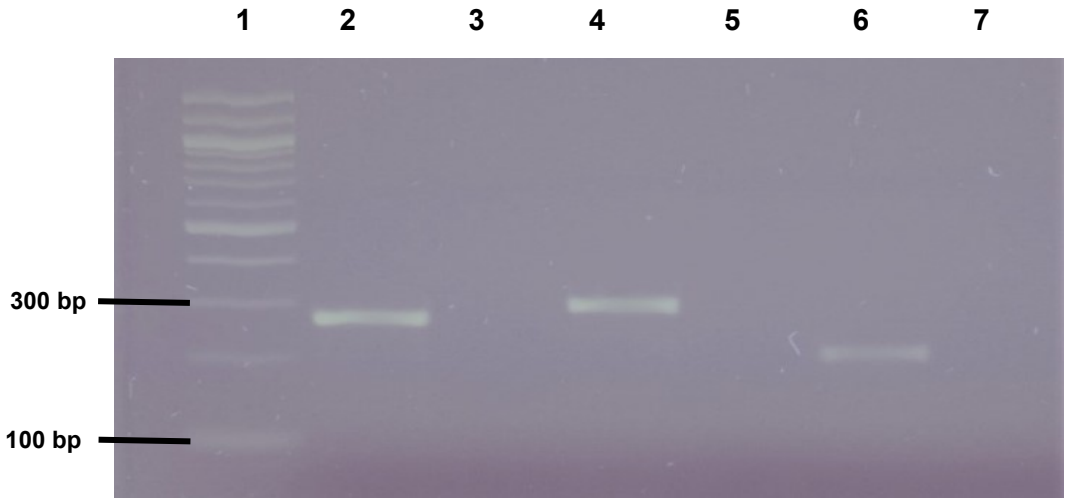
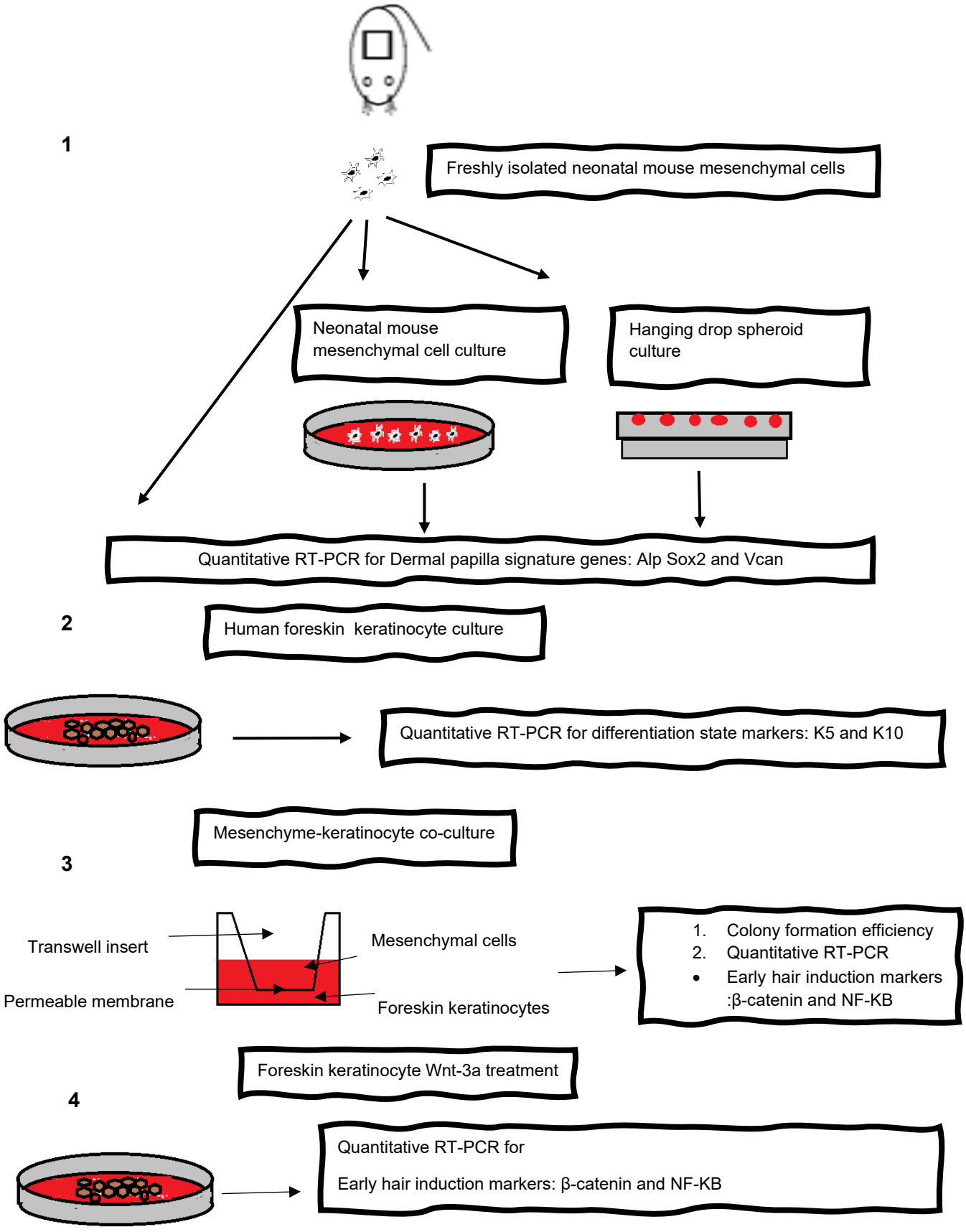


Figure 8: Agarose gel electrophoresis diagram of dermal papilla signature markers primers on mouse embryonic stem cells. Vcan (212 base pairs(bp)), migrated the furthest, Sox2 (289 bp) migrated the least and alkaline phosphatase (267 bp) is in the middle. Both bands are between 100 and 300 base pairs bands of the 100 bp ladder.

Lane 1:	100 bp ladder
Lane 2:	Alp 267 bp
Lane 3:	NTC
Lane 4:	Sox2 289 bp
Lane 5:	NTC
Lane 6:	Vcan 212 bp
Lane 7:	NTC

2.3 Methods Summary



3 Chapter 3: Results

3.1 Neonatal mouse mesenchyme cell culture and expression of dermal papilla markers

The aim of the study was to determine whether *in vitro* propagated neonatal mouse mesenchyme would be able to elicit expression of markers related to hair follicle induction in human neonatal foreskin keratinocytes. Neonatal mouse mesenchymal cells were chosen because they are primitive and are capable inducing hair follicles formation when combined with mouse skin keratinocytes (Zheng, et al., 2005; Kageyama, et al., 2018).

The first set of experiments were aimed at determining the characteristics of the dissociated neonatal mouse mesenchymal cells, including morphology, growth kinetics and gene expression over subsequent passages. The cells were obtained from the dermis of postnatal day 2 mice dorsal skin, dissociated and cultured in standard medium containing DMEM plus 10% FBS and antibiotics. The isolated cells rapidly adhered to the surface of the culture dish and after 24 hours they exhibited a mesenchymal-like morphology with a slightly flattened cell bodies and multiple processes (Fig. 9 arrows). Furthermore, they showed the characteristic aggregations of cells as they proliferate, as previously described for mouse dermal papilla cells cultured in medium containing foetal calf serum (Fig. 9 *) (Randall, et al., 1996).

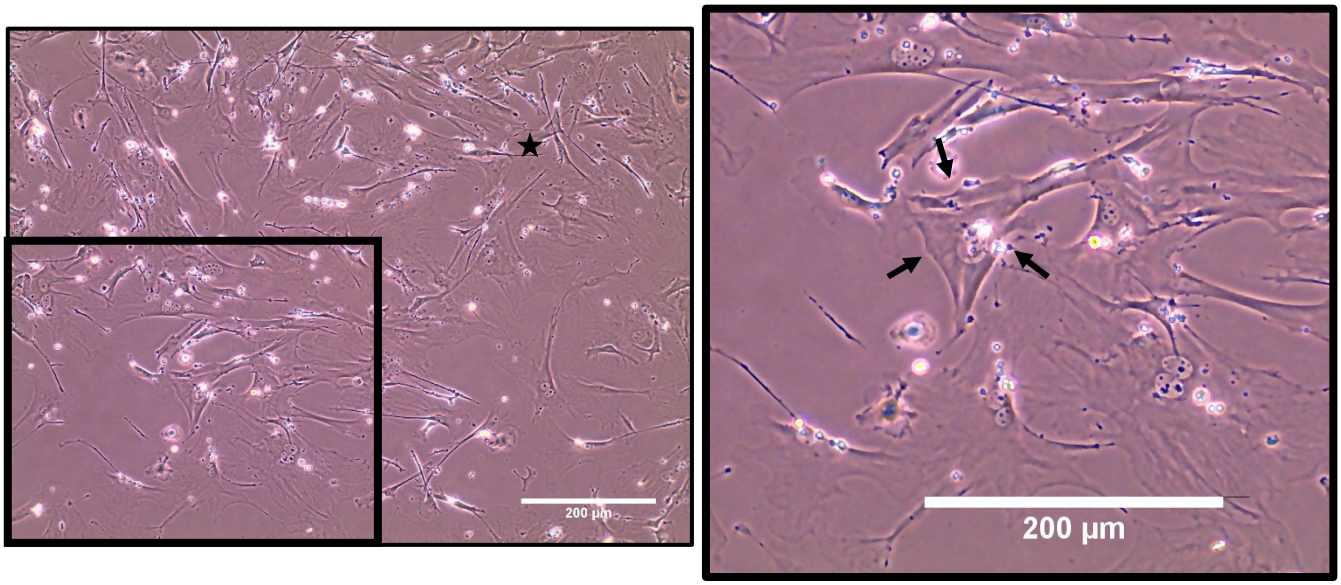


Figure 9: Neonatal mouse mesenchymal cells in culture. Neonatal mouse mesenchymal cells exhibited a mesenchymal-cell morphology with a slightly flattened cell bodies and multiple processes pointed by arrows. In confluency they showed the characteristic aggregations (star) of cells as they proliferate. (scale bar: 200 µm). 10 x Objective.

The main aim of growing cells in culture in bioengineering studies is to increase the number of cells with regenerative potential. To determine whether the dissociated neonatal mouse mesenchymal cells are able to proliferate in vitro, a growth curve was performed over a period of 4 days using cells at passage 2. The cells were plated in triplicate at a density of 24.25×10^4 per 60 mm cell culture dishes.

After 24 hrs the cell count was 18.66 x 10^4 showing a seeding efficiency of over 75%. The cells were in the lag phase until day 1 and started growing exponentially from day 2. By day 4 the growth rate had slowed down as they became confluent (Fig. 10). The calculated cell population doubling time for these neonatal mouse mesenchymal cells was 20 hrs. The growth curve demonstrated that the dissociated cells were able to proliferate and could be expanded in vitro for use in this study.

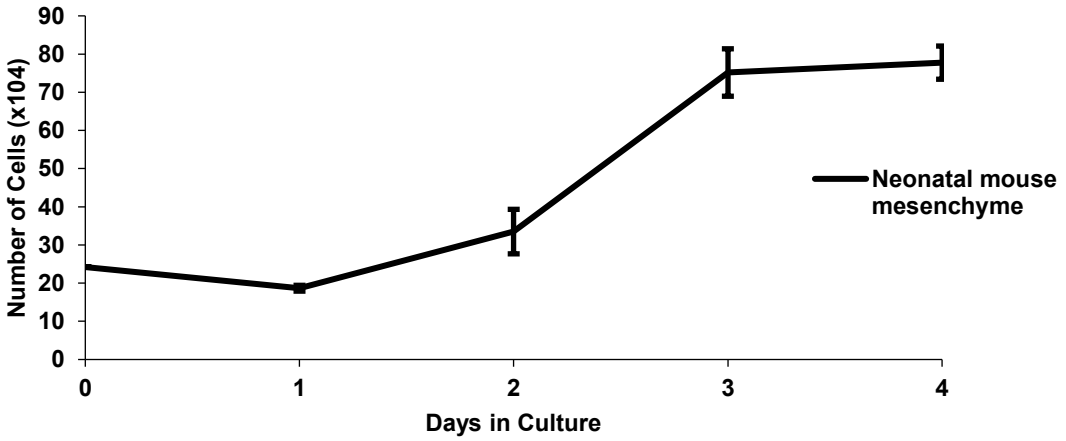


Figure 10: Growth curve of neonatal mouse mesenchymal cells over the period of 4 days. The seeding efficiency is at 75.26% as the cell count on the first day of culture was 18.67 x10^4. The cells recovered and they were in the lag phase from day 1 and grew exponentially from day 2. The cells plateaued on day 4. Each point represents mean value of three cell counts.

Several markers have been used to specifically identify dermal papilla cells, that are able to induce hair follicle formation when transplanted into the skin of SCID mice. Two of the markers, alkaline phosphatase (Alp) and Sox2, are typical stem cell markers. Alp has been shown to be expressed in cells that have hair induction capabilities (Driskell, et al., 2009). Sox2 has been shown to be expressed in dermal condensates at E14.5 and continues to be expressed in mouse adult tissue that have regenerative potential (Driskell, et al., 2009). A third marker, versican (Vcan) is a differentiation marker and it is highly and specifically expressed in the aggregated dermal papilla cells (Elliott, et al., 2016).

The first experiment was aimed at determining whether in this study the freshly isolated neonatal mouse mesenchymal cells from skin express the above dermal papilla signature markers. Total RNA was extracted from single cells isolated from the dermis of E18.5, E19.5 and postnatal day 0 dorsal skin and reverse transcribed into cDNA. The cDNA was used to perform quantitative RT-PCR using the StepOne Plus (Applied Biosystems).Gene expression levels were analysed using the $2^{-\Delta\Delta C_T}$ method and normalised to the reference gene Mus Glucuronidase B (mGus). Mouse embryonic stem cells (mES) (The mES mRNA used here was stored at -80º C and it was extracted from mES cultured

on irradiated mouse embryonic fibroblasts (iMEFs) feeders) was used as an internal control since they are known to express Sox2 and Alp.

Results show that Alp expression levels were high in mES as compared to E18.5 neonatal mouse mesenchymal cells and thereafter the expression was increased from E19.5 to postnatal day 0 (Fig.11 A). Sox2 gene was highly expressed in mES cells as compared to neonatal mouse mesenchymal cells and was reduced during the progression of hair follicle development, from E18.5 to postnatal day 0 cells (Fig. 11 C). In contrast, Vcan which is usually expressed in aggregating cells was expressed at very low levels in mES and there was no difference in Vcan gene expression in E18.5, E19.5 and postnatal day 0 cells (Fig. 11 B).

Having determined that neonatal mouse mesenchymal do express dermal papilla signature genes, the next set of experiments were aimed at determining whether cultured and passaged neonatal mouse cells express dermal papilla signature markers.

The results presented here are a summary of 6 independent experiments and Passage 0 to 2 for Alp and Vcan; and Passage 0 to 4 for Sox2, were not included because they were diluted, but the expression represents the trend. Mouse embryonic stem cells were used as a control for all the samples. The standard deviation (SD) was obtained from duplicates of a single experiment.

As can be seen, both, Alp and Sox2 gene expression levels were reduced to non-detectable levels in passaged neonatal mouse mesenchymal cells as compared to freshly isolated neonatal mouse mesenchymal cells (Fig. 11 A and C). Vcan gene was slightly reduced in cultured cells and its expression varied with change in passage number, even though ~11 x 10^4 cells were plated in 6-cm tissue culture plates and RNA extracted at day 4 in all experiments (Fig. 11 B). These results are consistent with previous studies which show that dermal papilla cells lose their inductive potential by *in vitro* propagation (Horne & Jahoda, 1986).

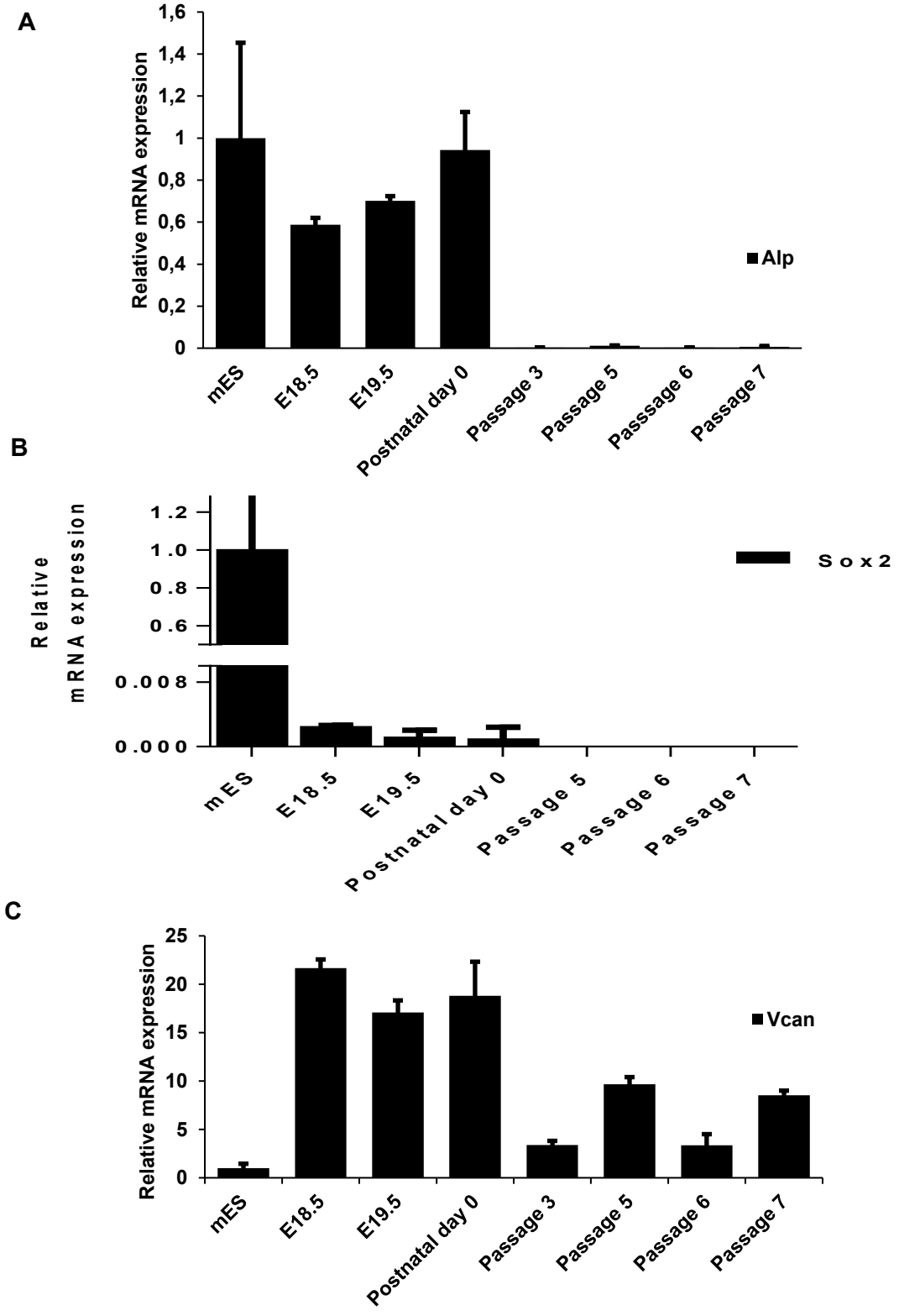


Figure 11: Expression of dermal papilla markers in mouse mesenchyme during embryonic development and over subsequent passages. (A) Alp was upregulated during embryonic hair follicle development in E18.5, E19.5 and postnatal day 0 cells and reduced to non-detectable level in passaged neonatal mouse mesenchymal cells. (B) Sox2 was downregulated during the progression of hair morphogenesis and reduced to non-detectable level in passaged cells. (C) Vcan was maintained from E18.5 until postnatal day 0 and it was downregulated and varied in different passages. (SD was obtained from duplicates of a single experiment).

3.2 Effect of cell density on the expression of dermal papilla signature markers

In the above experiment, the neonatal mouse mesenchymal cells were cultured *in vitro* in a monolayer and cells were harvested at about 70% confluency. However, *in vivo* mesenchymal cells would be in contact with each other in order to be inductive, therefore they need to be cultured in a way that resembles *in vivo* dermal papilla (Holbrook & Odland, 1975).

The next experiment was therefore designed to determine whether confluency (i.e. increasing cell-cell contact) affects expression of dermal papilla markers. Neonatal mouse mesenchymal cells were seeded at 0.5 x 10^4 cells/cm^2, 0.8 x 10^4 cells/cm^2 and 1.6 x 10^4 cells/cm^2 and cultured for 4 days, as can been seen in Fig. 12 A a-c, cells seeded at different concentrations were at different confluency.

When gene expression was measured, Alp gene expression was increased from 0.5 x 10^4 cells/cm^2 to 0.8 x 10^4 cells/cm^2 and thereafter remained constant. Sox2 gene expression was uniform from 0.5 x 10^4 cells/cm^2 to 0.8 x 10^4 cells/cm^2 and thereafter it was increased in 1.6 x 10^4 cells/cm^2. Versican gene expression is upregulated in a concentration dependent manner from 0.5 x 10^4 cells/cm^2 to 1.6 x 10^4 cells/cm^2 (Fig. 12 B). The results suggest that increased cell-to-cell contact is crucial for maintenance of the expression of dermal papilla signature markers of neonatal mouse mesenchymal cells.

Another approach to test the role of cell-cell contact was to create 3D spheres using a hanging drop culture system. Therefore, in the next experiment neonatal mouse cells were aggregated using the hanging-drop culture system in order to maximise cell-to-cell contact. The hanging drop spheroid model is based on a surface tension-based technique and the interaction between surface tension and gravity that makes a convergence of liquid drops (Lin, et al., 2016). Drops of 10µl DMEM that contain 3000 neonatal mouse mesenchymal cells were pipetted on inverted culture plate lid (Fig. 13 A).The lid was reverted and cultured for 48 hours at 37º C 5% CO_2 (Fig. 13 B and C).The cells clustered and formed spheroids within 24 hours (Fig. 13 D, E (zoomed)). Fig. 13 F shows a spheroid formed using the Agarose gel method for spheroids. For each analysis, two 6-cm culture plates comprising approximately 30 spheroids were used to extract RNA for qPCR analysis. Quantitative real-time PCR was carried out to analyse the expression of dermal papilla signature markers in mouse mesenchyme spheres. The expression level was assessed by relative expression level based on adherent neonatal mouse mesenchymal cells at high confluency. The experiment was carried out twice in duplicate and the results presented are an average from both experiments.

All markers were upregulated in neonatal mouse mesenchymal cell spheroids as compared to 2D cultured mesenchymal cells (Fig. 13 G). Alp was increased 50x in spheroids and there was a 4+-fold increase of Sox2 expression. The results showed 2-fold increase in Vcan expression. The results suggest that aggregation of neonatal mouse mesenchymal cells results in a reactivation and expression of genes that are linked to inductive ability, i.e. Sox2, Alp and Vcan.

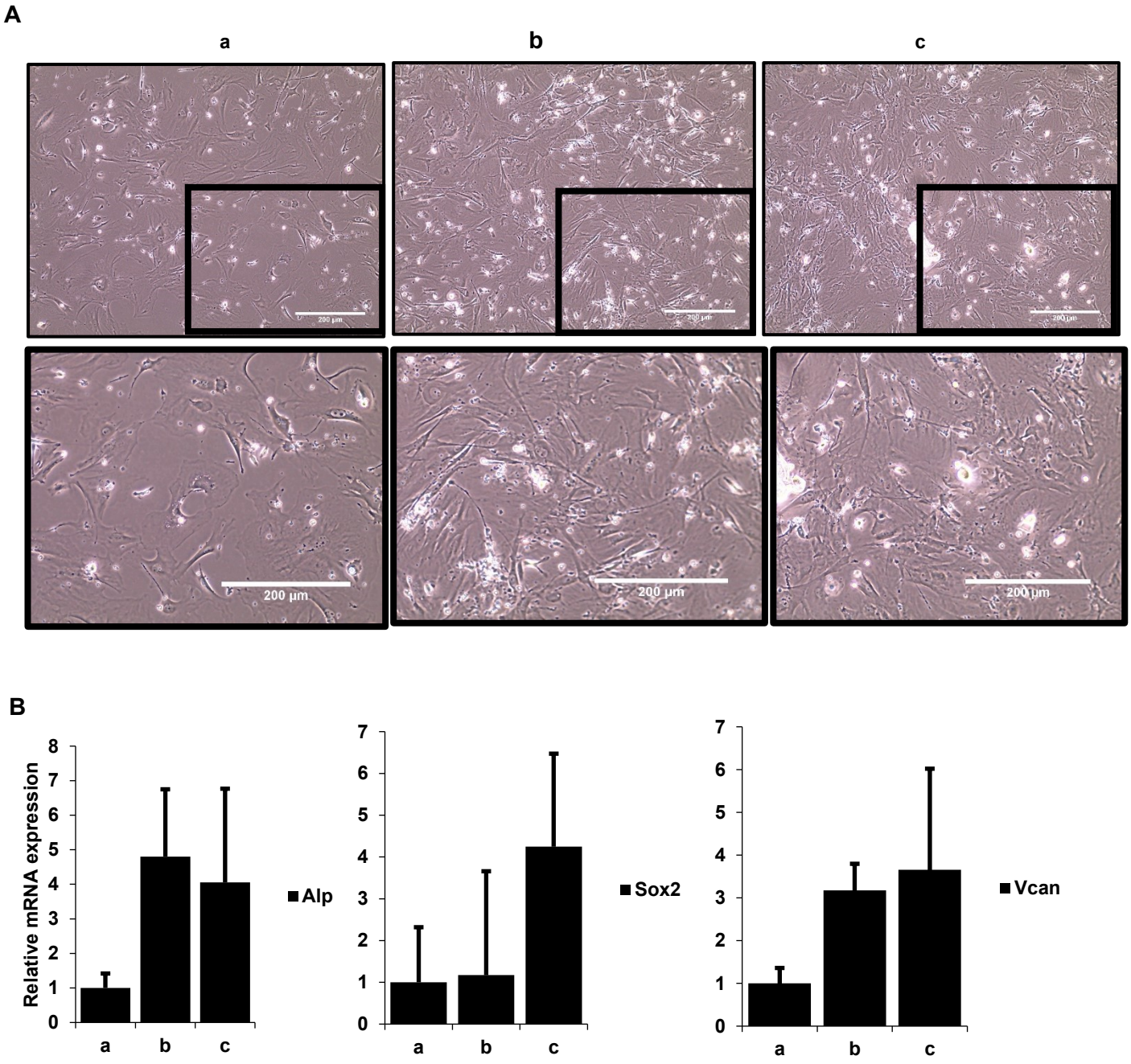


Figure 12: Effect of cell density on the expression of dermal papilla signature markers. Cells were seeded at 0.5 x 10^4 cells/cm^2 (a), 0.8 x 10^4 cells/cm^2 (b) and 1.6 x 10^4 cells/cm^2 (c) . (A) Confluency of neonatal mouse mesenchymal cells at day 4. (B) Relative mRNA level of dermal papilla signature markers in all cell densities. Alp expression was upregulated in 0.8 x 10^4 cells/cm^2 and 1.6 x 10^4 cells/cm^2. Sox2 was upregulated in 1.6 x 10^4 cells/cm^2. Vcan was upregulated in a concentration dependent manner. The data represent the mean of duplicates of a single experiment. SD was conducted on using duplicates.

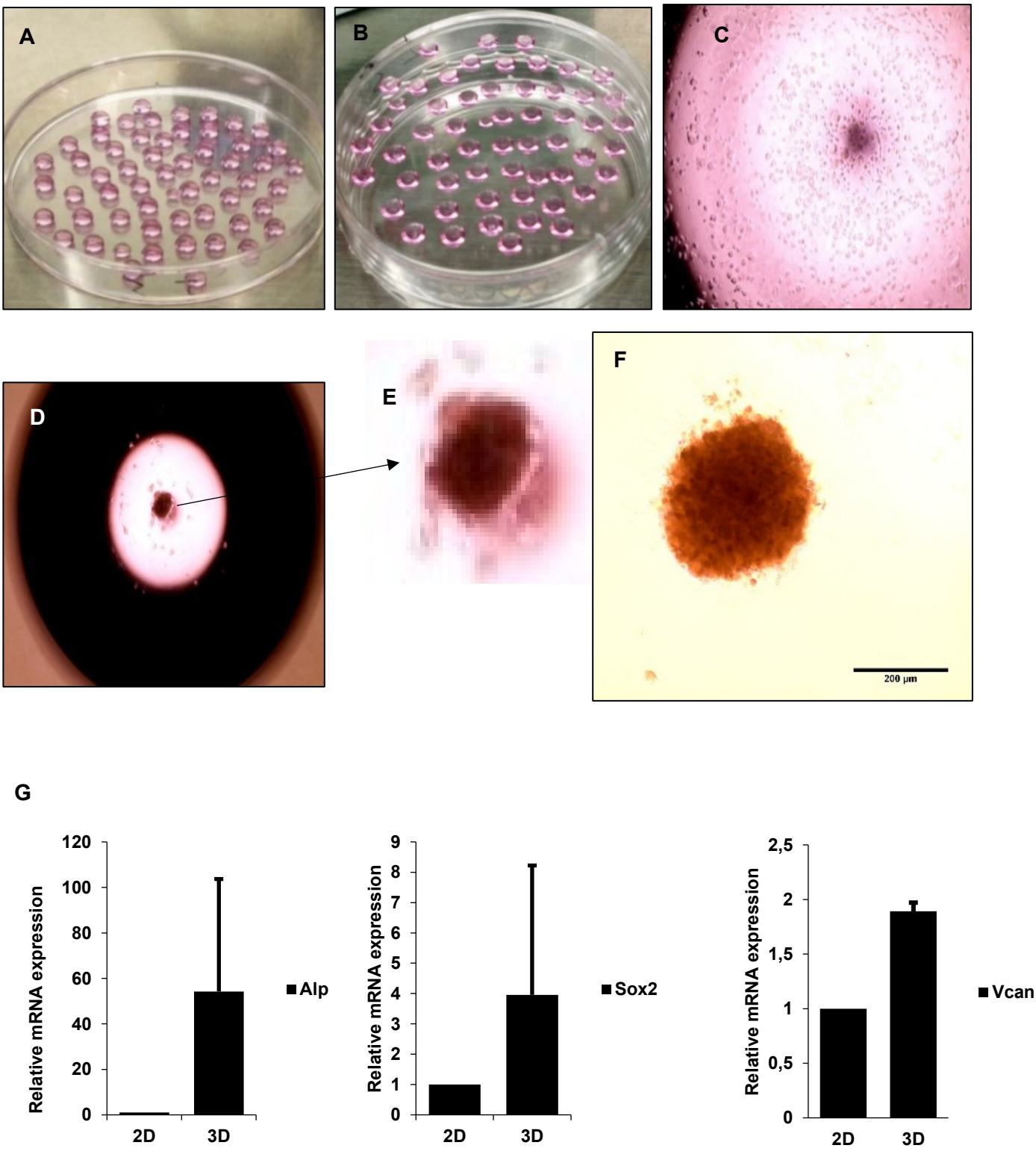


Figure 13: Expression of dermal papilla signature genes in neonatal mouse mesenchymal cells cultured in spheroids. (A) Mesenchymal cells were cultured in 10 µL droplets of DMEM containing 3000 cells on inverted lid. (B) droplets facing down (C) closer view of cells in the droplet. (D) Spheroids in 48 hours. (E) closer view of spheroids. (F) Spheroid formed using the Agarose gel method for spheroids. (G) Quantitative RT-PCR was performed from RNA isolated from spheroids (3D) mesenchyme. Gene expression is relative to monolayer cultured mesenchyme (2D). Dermal papilla signature markers, Alp, Sox2 and Vcan were all upregilated in spheroid cultures. (Scale bar: 200 µm). (N=2 for Alp and Vcan) and (N=1 for Sox2).

3.3 Human foreskin keratinocyte culture and determination of differentiation markers

Having established that mouse mesenchymal cells express markers that relate to inductive potential, the next step was to establish cultures of keratinocytes that would potentially be responsive to inductive signals. In this study, primary cultures of human foreskin keratinocytes were tested.

Keratinocytes were isolated from human foreskins obtained during child circumcision and cultured in KSFM as described in Materials and Methods. To determine the proliferative potential of keratinocytes, 55 x 10^4 cells were seeded in a 6-cm plate in KSFM medium. The medium was changed after three days to allow cells to adhere to the plate. At 24 hours, cells were sparse, and many single cells could be seen with few cell clusters visible (Fig. 14 A arrow). Colonies started forming on the second day (Fig. 14 B arrows) and were merging on the fourth day (Fig.14 C). On the seventh day the colonies eventually fused, forming a monolayer of epithelial cells, demonstrating a typical cobblestone morphology (Fig. 14 D). The ability to form a confluent monolayer indicates that these keratinocytes are proliferative and can be used for subsequent experiments.

In normal skin the basal layer is composed of undifferentiated keratinocytes which are characterised by their high proliferative potential and expression of Keratin 5 (K5) (Premio, et al., 1999). In contrast, high expression of the early differentiation marker, Keratin 10 (K10) correlates to an inhibition of cell proliferation of keratinocytes *in vivo* and *in vitro* (Premio, et al., 1999). Therefore, to determine the state of differentiation of cells, the markers keratin 5 and 10 were used. Ideally cultures expressing higher levels of K5 than K10 would be suitable for inductive experiments.

RT-qPCR was performed on keratinocytes harvested after 7 days of culture and B-actin was used as an internal control. Tests were carried out on passage 2 as well as passage 3 cells, and K5 and K10 gene expression levels were compared. Results showed that K5 was expressed at a higher level as compared to K10, which was minimal (Fig. 15). The results further confirm that the cultured keratinocytes are not differentiated and can be used for subsequent experiments on early induction.

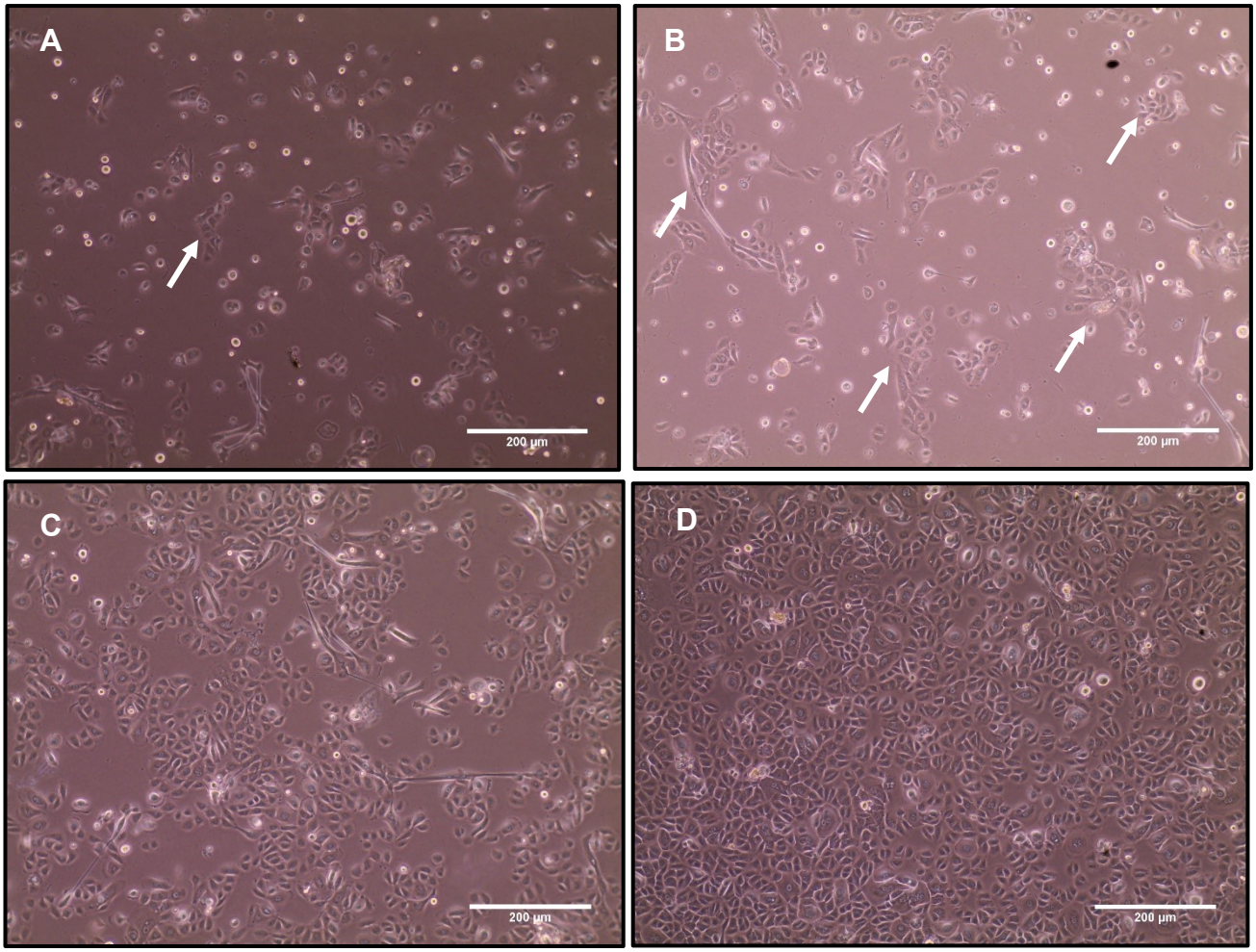


Figure 14: Human foreskin keratinocytes in culture. (A) at 24 hours showing single cells. (B) Keratinocytes at 2 days showing colony formation. (C) At 4 days, colonies were fusing. (D) At day 7 the confluent monolayer demonstrated a cobblestone morphology, typical of epithelial cells. (Scale bar: 200 µm). 10x objective.

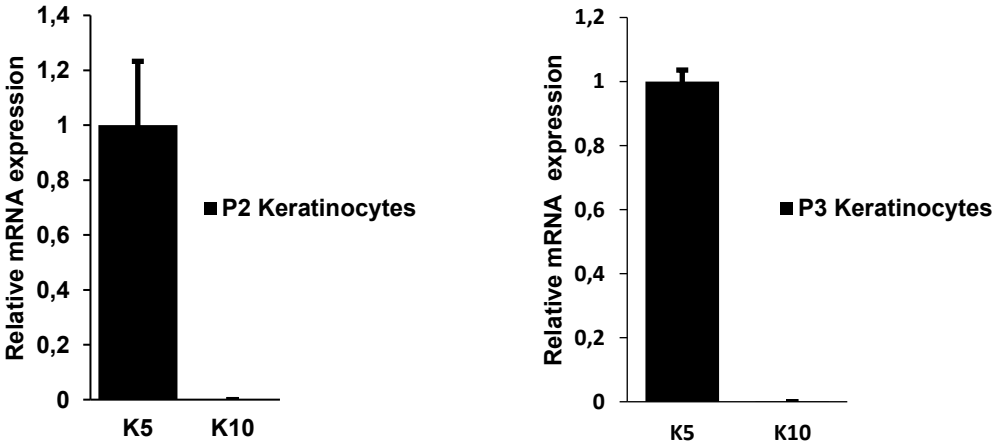
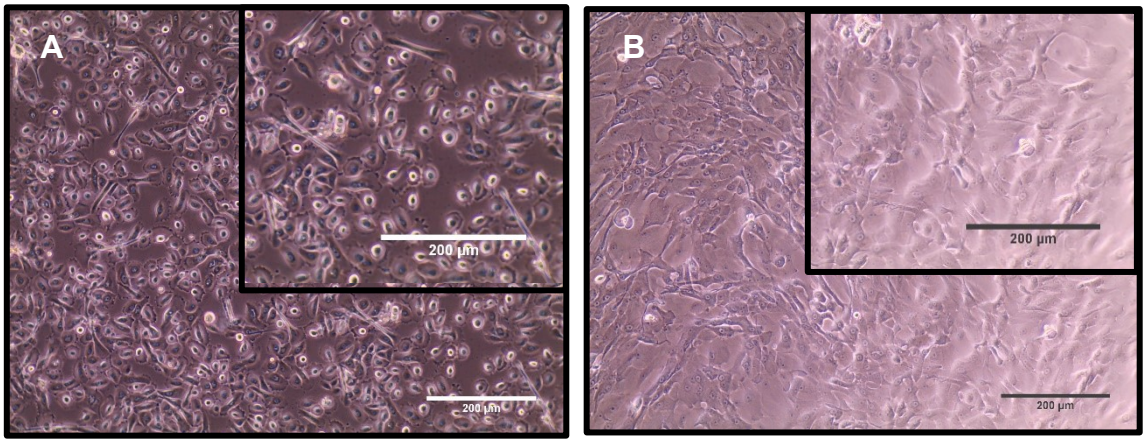


Figure 15: Expression of differentiation markers in cultured keratinocytes. RT-PCR for K5 and K10 in keratinocytes. The relative expression was normalised to the reference gene B-actin. The data represent the means ± SD of 2 experiments in duplicate. K5 was upregulated in keratinocytes as compared to K10.

3.4 Evaluating the effect of neonatal mouse mesenchymal cells on human foreskin keratinocytes in a co-culture

In order to carry out induction experiments it was necessary to coculture mesenchymal cells with keratinocytes. It was therefore necessary to determine which culture medium will be best suited for both cell lines since mesenchymal cells were normally cultured in DMEM with 10% FBS while keratinocytes were cultured in specialised KSFM medium. To determine which medium combination would be suitable for keratinocytes in a co-culture system, keratinocytes were first cultured in a medium that comprised basal DMEM plus 10% FBS/KSFM (1:1) and as a control, keratinocytes were cultured in KSFM only. The KSFM cultured keratinocytes exhibited the typical cobblestone morphology of epithelial cells in culture (Fig. 16 A), while the keratinocytes that were cultured in DMEM plus 10% FBS/KFSM took on a more fibroblast-like morphology (Fig. 16 B). Furthermore, RT-qPCR was performed to determine the differentiation state of the cells in DMEM plus 10% FBS/KSFM (1:1) as compared to the culture in KSFM alone. The results showed that K5 gene expression levels were slightly reduced and K10 gene expression levels were halved (Fig. 16 C). The results suggest that adding serum to the medium alters the characteristic of keratinocytes. Following this finding serum was removed from all subsequent experiments that involved primary keratinocyte cultures.



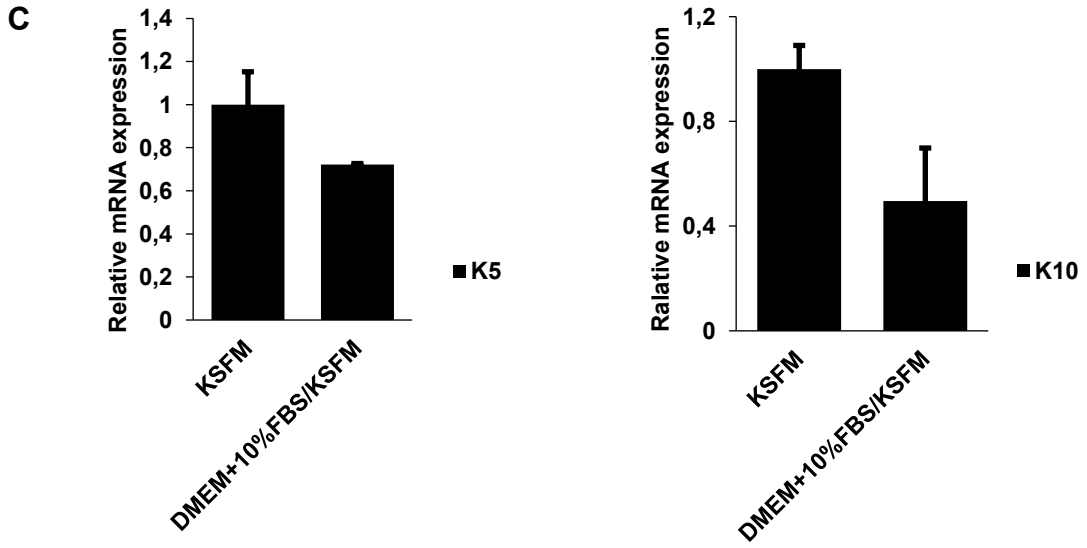


Figure 16: Culture conditions suitable for neonatal mouse mesenchymal cells and foreskin keratinocytes. (A) KSFM cultured neonatal foreskin keratinocytes. (B) Neonatal foreskin keratinocytes cultured in KSFM:DMEM supplemented with 10% FBS. (C) Quantitative RT-PCR for differentiation markers, K10 and K5. Relative expression was normalised to the reference gene B-actin. SD was obtained from duplicates of a single experiment. K5 expression was slightly reduced in DMEM plus 10% FBS/KSFM keratinocytes. K10 expression levels was halved in DMEM plus 10% FBS/KSFM keratinocytes. (Scale bar: 200 μm).

Having established the medium that is suitable for keratinocyte culture, the Transwell co-culture system was used to investigate the effect of neonatal mouse mesenchymal cells on keratinocytes. This system allows growth of two cell types using inserts with permeable membranes that allow diffusion of secreted soluble factors (Renaud & Martinoli, 2016).

In the preliminary co-culture experiments, human foreskin keratinocytes were cultured in 3.5 cm diameter dishes, and the mesenchymal cells were cultured on 1.2 cm diameter, 0.4 μm pore size inserts that were placed in the centre of the culture well (Fig. 17 A). It was observed that keratinocytes beneath the insert were proliferating more than those at the edges of the plate, suggesting that diffusible factors from mesenchymal cells had proliferative effect on keratinocytes.

To test this hypothesis, the next step was to design an experiment that can quantitate this observation. For this experiment, inserts containing 3 x 10^4 mesenchymal cells were immersed in 6-well cluster plates seeded with 18 x 10^4 keratinocytes on Geltrex™. The culture medium comprised basal DMEM (without FBS)/KSFM (1:1). After 2 days the developing keratinocyte colonies were counted manually on the tissue culture plate as described in Materials and Methods. Only colonies consisting of >3 cells were considered in the count. As shown in Figure 17 B, the cells in the middle, which are underneath the insert formed more colonies as compared to the ones at the periphery which (Fig. 17 C). Keratinocytes growing immediately under the 1.2 cm insert proliferated more as compared to the keratinocytes at the edges of the 3.5 cm plate (Fig. 17 D). Colonies consisting of 3 or more cells were minimal at the edges while in the middle, there were as many as 35. These results suggest that diffusible factors from mesenchymal cells promote colony forming potential in cultured keratinocytes, indicating they could play an important role in hair follicle morphogenesis.

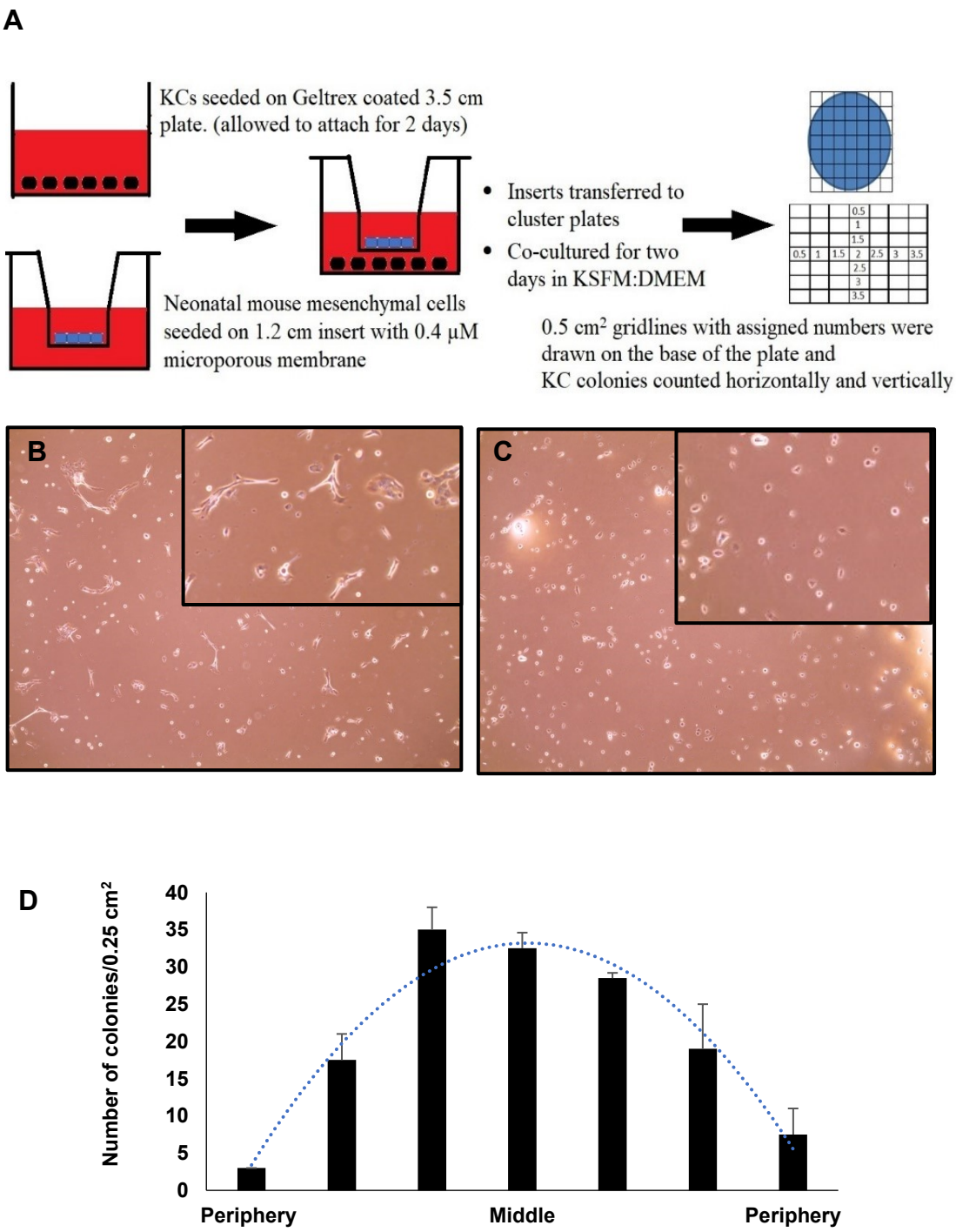


Figure 17: The effect of neonatal mouse mesenchymal cells diffusible factors on keratinocytes colony formation. (A) Coculture method for colony formation analysis. (B) Keratinocytes colonies in the middle of the cluster plate. (C) single cells could be seen on the periphery of the plate. (D) Number of keratinocyte colonies over the diameter of a 6-well cluster plate of the horizontal assigned axis. Most colonies were growing in the middle of the cluster plate as compared to in the periphery. The experiment was performed in sextuplicate and the SD was obtained from the means.

3.5 Evaluating the effect of neonatal mouse mesenchymal cells on HaCaT cells in a co-culture

Due to complications encountered when expanding primary keratinocytes, it was necessary to conduct the next series of experiments with immortalised keratinocytes (HaCaT cells). The problems were as follows: (1) After sub-culturing and freezing the thawed keratinocytes seeding efficiency was lower than 50%, (2) Keratinocytes took 4 to 5 weeks to reach confluency and (3) in some cultures, cell proliferation ceased completely (i.e. cells became quiescent) as previously described. For these reasons HaCaT cells were used as a substitute to avoid delay while waiting for keratinocytes to reach confluency. HaCaT keratinocyte cell line is a spontaneously immortalised line and one of its advantages is that it doesn't need specialised keratinocyte growth medium (Boukamp, et al., 1988; Chio, et al., 2017).

To determine the effect of neonatal mouse mesenchymal cells on the expression of markers of early hair induction in HaCaT cells, co-culture was carried out as follows: HaCaT cells were first plated at a density of 10 x 10^4 cells per well on 12-well cluster plates in DMEM plus 10% FBS, separately. Mesenchymal cells were seeded in sextuplicate at 1 x 10^4 into 12-mm inserts and allowed to attach for two days. The inserts were then transferred to the HaCaT cells in 12-well cluster plates and both were cultured in DMEM plus 10% FBS. Medium change was done on the second day of co-culture and total RNA for quantitative RT-PCR was extracted on the 4^{th} day.

To determine whether diffusible factors from neonatal mouse mesenchymal influence HaCaT cells, quantitative RT-PCR for Wnt/β-catenin signalling downstream target genes and differentiation markers was carried out. Quantitative RT-PCR results showed that, β-Catenin gene expression was increased 4-fold in co-cultured HaCaT cells. NF-KB gene expression was slightly increased in co-cultured HaCaT cells. There was 7-fold increase in the expression of basal layer marker K5 in co-cultured HaCaT cells. There was 4.5-fold increase of the early differentiation marker, K10 in co-cultured HaCaT cells (Fig. 18).

In the second experiment the number of HaCaT cells were reduced to 5 x 10^4 because in the first experiment they grew and reached confluency too quickly. In this experiment total RNA for quantitative RT-PCR was extracted when the cells were at 80% confluency. There was no difference in the morphology of co-cultured HaCaT cells and controls.

Quantitative RT-PCR results showed that, β-catenin gene expression was downregulated in co-cultured HaCaT cells. In contrast, there was a 1.6-fold increase of NF-KB gene expression. There was a slight reduction of K5 gene expression, however, K10 gene expression was increased 9-fold (Fig. 19). The results suggest that diffusible factors from neonatal mouse mesenchymal cells influenced HaCaT cells, as seen by an increase in expression of Wnt/β-catenin signalling target genes

and differentiation markers in the first experiment even though β-catenin and K5 were not increased in the second experiment.

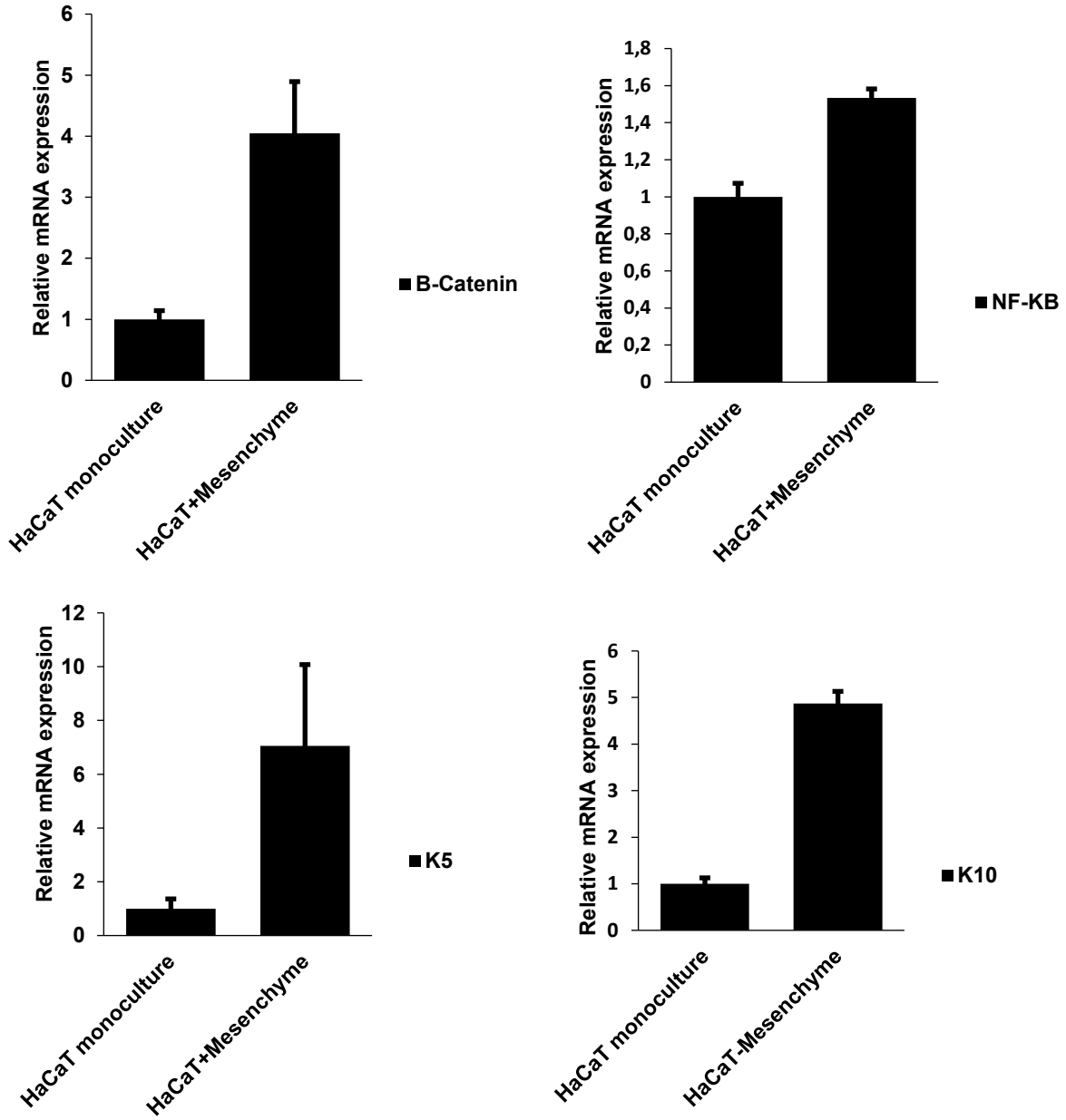


Figure 18: HaCaT-neonatal mouse mesenchymal cells co-culture experiment 1. HaCaT cells were plated on 12-well cluster plates at 10 x 10^4 cell density and were co-cultured with 1 x 10^4 neonatal mouse mesechymal cells on 12-mm inserts for two days in DMEM 10% FBS. Total RNA was extracted to perform quantitative RT-PCR for markers of early hair induction and differentiation markers. Relative expression was normalised to the reference gene B-actin. There was 4-fold increase in B-Catenin expression in HaCaT co-cultures. NF-KB expression levels were slightly increased in HaCaT co-cultures. There was a 7-fold increase in K5 expression in co-cultured HaCaT cells. K10 expression levels were increased 4.5-fold in HaCaT co-cultures. (N=1) SD was obtained from duplicate values of a single experiment.

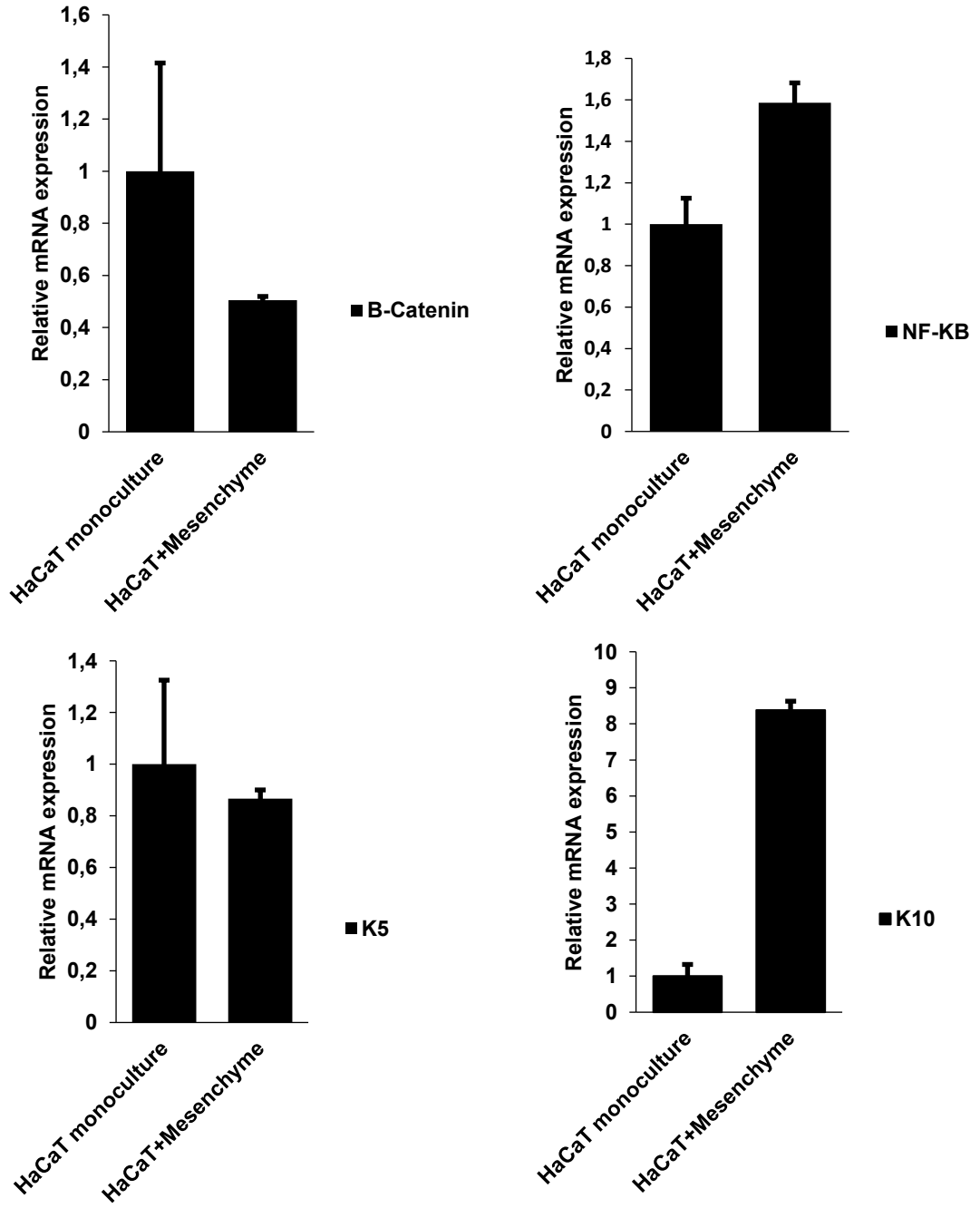


Figure 19: HaCaT-neonatal mouse mesenchymal cells co-culture experiment 2. HaCaT at 5 x10^4 cells per well in a 12-well cluster plate were co-cultured with 1 x 10^4 neonatal mouse mesenchymal cells in 12-mm inserts for 4 days. Total RNA was extracted to perform quantitative RT-PCR for markers of early hair induction and differentiation markers. Relative expression was normalised to the reference gene B-actin. B-Catenin expression was halved in co-cultured HaCaT cells. NF-KB was slightly upregulated in co-cultured HaCaT cells. K5 gene expression was unchanged. There was an 8.5-fold increase in K10 expression in co-cultured HaCaT cells. (N=1) SD was obtained from duplicate values of a single experiment.

3.6 Effect of Wnt-3a on human foreskin keratinocytes

Wnt-3a is the ligand of the canonical Wnt signalling pathway, it binds to Frizzled (Fz) receptors and its co-receptors, low density lipoprotein receptor-related protein (LRP-5) or LRP6 which result in the inhibition of β-catenin phosphorylation and its subsequent degradation by glycogen synthase kinase. The stabilised β-catenin translocates to the nucleus where it acts as a transcription factor for LEF/TCF, as discussed above. Wnt-3a has previously been shown to activate the Wnt/β-catenin signalling pathway resulting in the expression of a stabilised form of β-catenin in keratinocytes during hair follicle induction (Gat, et al., 1998; Fu & Hsu, 2013).

This next experiment was aimed at determining whether the Wnt/β-Catenin signalling pathway could be activated by culturing keratinocytes in medium containing Wnt-3a. Conditioned medium containing Wnt protein was obtained from L Wnt-3a (ATCC®) CRL-2647™) cells that biologically synthesize active Wnt-3a protein. The L Wnt-3a were grown to 70% confluency in DMEM plus 10% FBS. For collection of conditioned medium, the medium was replaced with serum-free DMEM/F12 as described in Materials and Methods. For treatment Wnt-3a conditioned medium was diluted 50/50 with KSFM medium. As a control, the conditioned medium was obtained from control L cells (ATCC CRL-2648), that do not contain the expression vector. Keratinocytes were cultured to 70% confluency in 3.5 cm culture plates and thereafter treated for 3 days. Total mRNA was extracted to perform quantitative RT-PCR of Wnt/β-catenin signalling pathway downstream genes.

Lef1 gene expression levels were slightly increased in Wnt-3a treated foreskin keratinocytes, while, both β-catenin and NF-KB gene expression levels were slightly reduced in Wnt-3a treated foreskin keratinocytes. With two of the three selected Wnt signalling downstream genes reduced following Wnt-3a treatment the, results may suggest that keratinocytes were not sensitive enough to respond to Wnt-3a stimulation (Fig. 20), or it was because Wnt3a protein lost its stability since the treatment was done for 3 days (Tuysuz, et al., 2017). Perhaps, a chemical such as 6-bromoindirubin-3-oxime (BIO) that can activate the Wnt/β-catenin signalling pathway should have been used instead (Soma, et al., 2012).

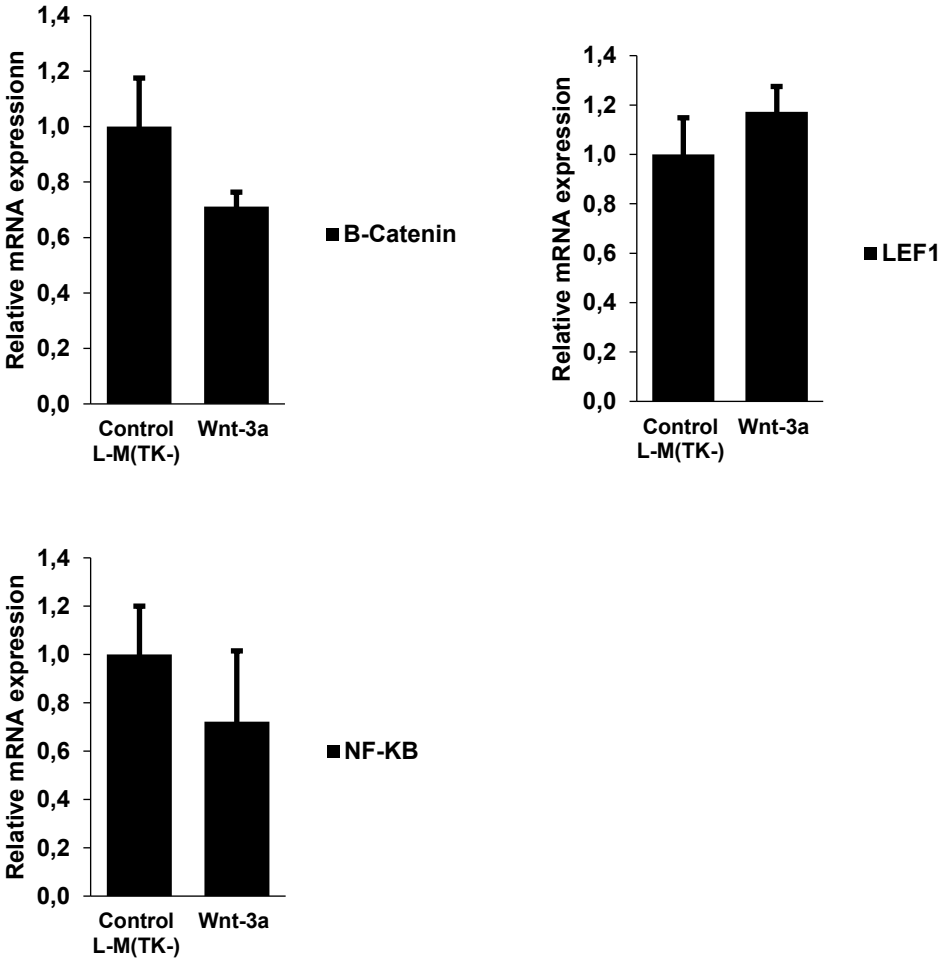


Figure 20: Expression of hair induction molecules in human foreskin keratinocytes cultured in Wnt3 conditioned medium. Total RNA for quantitative RT-PCR was extracted after 3 days of Wnt-3a treatment. B-Catenin and NF-KB expression levels were slightly reduced in Wnt-3a treated keratinocytes. Lef1 gene expression was slightly upregulated in Wnt-3a keratinocytes. (N=1) SD was obtained from duplicate values of a single experiment.

4 Discussion

Burn injuries, often caused by fire, resulting from the use of fuel in informal housing settlements remains a public health and medical challenge and according to a report by the WHO (World Health Organization , 2018), it costs South Africa an estimated amount of 26 million annually to take care of burn victims . Among other complications, the surviving patients suffer from infections of the exposed wounds and severe scarring which can lead to loss of or compromisation of limb function and devastating disfigurement. In addition, these thermal injuries result in the loss of skin appendages, including hair follicles and sebaceous and sweat glands. Efforts to improve treatment to create skin substitutes is ongoing across the world. Current treatment includes the use of split-thickness skin autografts harvested from healthy donor sites. However, in cases where the burns are extensive donor sites become limited. In this case keratinocytes can be obtained from the remaining skin tissue and expanded in culture to get considerable number of cells for wound coverage. The cells are then "seeded" onto a prepared wound bed. In these types of cases grafts improve wound coverage and healing to minimise (and therefore save lives) but the hair follicles and glands never reform (or regenerate) (Ronfard, et al., 2000). So far attempts to regenerate hair follicles have been carried out using animal cells and various methods wherein epithelial and mesenchymal components were enzymatically separated, recombined and engraftmented in nude mice. These experiments have generated positive results, even when non-hair forming epithelium, such as corneal epithelium was used (Reynolds & Jahoda, 1992; Pearton, et al., 2005; Zheng, et al., 2005; Kageyama, et al., 2018). However, these experiments have not been reported using expanded human epithelial cells and as far as can be established , the induction of hair follicles has not been achieved as yet.

The two specific questions that are addressed in this dissertation with respect to the above issue are:

1) What conditions need to be established to generate sufficient quantities of mesenchymal cells so that they retain inductive potential and,
2) Are cultured human foreskin keratinocytes competent to respond to signals from instructive dermis (mesenchyme) and do they express genes that are relate to hair follicle formation. Part of this question is whether committed cells from glabrous skin can de-differentiate and then re-differentiate into hair bearing skin.

4.1 The role of mesenchymal cells aggregation in relation to hair follicle induction

The key feature or marker of the dermal cells during early hair follicle induction is that they begin to aggregate in clusters in response to Wnt signals from the epidermis. In addition to forming aggregates, these dermal cells express a set of typical genes including alkaline phosphatase, Sox2 (both stem cell markers) and versican (a cell adhesion marker). Expression of the typical genes are used to predict the initiation of hair follicle morphogenesis during embryogenesis and to differentiate hair forming mesenchymal cells from other dermal cells in adult tissue.

The first set of experiments in this study revealed that intact dermis expressed Alp, Sox2 and versican, similar to what was found by Driskell *et al.,* (2009). In brief, , Alp, a typical marker of hair inductive potential, increased from day E18.5 to postnatal day 0. This differs slightly from Driskell *et al.,* (2009), who found the peak at E18.5 and a major down regulation at postnatal day 2, but because this study did not include P2 & P4, one cannot be sure that the results are different. Sox2, a typical stem cell marker was reduced from E18.5 to postnatal day 0, this corresponds with normal development, where maximum stem cell potential falls after hair follicles have been induced between E14.5 and E18.5. This also aligned to what was found by Driskell. Versican, an adhesion molecule which is expressed in normal developing hair dermis, marks the commencement of cell aggregation. In this study, versican expression remained constant from E18.5 to postnatal day 0, however this doesn't give insight of the time that mesenchymal cells start to aggregate, because E14.5 was not included in this study.

The next step of this work was to assess the expression of the above markers during culture of mesenchymal cells. Both stem cell markers, Alp and Sox2 reduced to non-detectable levels in cultured cells. Versican was slightly reduced and the expression was random at different passages. This is similar to the results found by Ohyama *et al.,* (2012), whereby gene expression of intact human dermal papilla and cultured dermal papilla cells were compared. They found that, Alp, versican and other dermal papilla signature genes were significantly reduced in cultured dermal papilla cells. It is further supported by Osada *et al.,* (2007), whereby dermal papilla cells lose versican gene expression following subsequent passaging. The results build on the existing knowledge of mesenchymal cells and confirm that mesenchymal cells lose their hair follicle inductive potential following *in vitro* propagation. It is evident that one of the reasons for the loss of markers would be due to the fact that cells are not grown in 3D cultures, but in 2D culture. It was therefore hypothesised that if cells are forced to "cluster", they might retain higher levels of dermal papilla gene expression. In this study, there were two approaches to doing this.

First, Alp, Sox2 and versican gene expression levels were increased when cell density was increased from 0.5×10^4 cells/cm^2 to 0.8×10^4 cells/cm^2 and 1.6×10^4 cells/cm^2 at day 4 of culture. The result indicates that it is crucial to maintain mesenchymal cell-to-cell contact to preserve the hair follicle inductive potential of mesenchymal cells since the cells that when the cells were brought closer together there was an increase in expression levels of dermal papilla signature markers. Similar results were obtained when mesenchymal cells were forced to cluster in spheroids by the hanging-drop culturing method, thereby mimicking the normal process of clustering. This is similar to that reported by Jacobson (1966) who showed that that dispersal of mouse mesenchyme condensates by X-irradiation during vibrissa follicle development leads to the disruption of hair follicle morphogenesis (Jacobson, 1966).

The upregulation of versican by clustering is consistent to what was reported by Miao *et al.,* (2014). However, there is an open question on whether the upregulation of versican is causative or a consequence of clustering, Feng *et al.,* (2010) found that versican is needed for clustering because versican ablation with small interfering RNA resulted in loss of aggregative ability of mesenchymal cells. This needs to be further investigated using experimental designs that clearly separate cause from consequence.

Alp has also been implicated in the condensation of mesenchymal cells during hair follicle formation. Pearton *et al.,* (2005), showed that alkaline phosphatase activity appeared when cells have started clumping in the dermis of developing mouse embryo, suggesting that it gets activated by cell-to-cell contact.

It is not clear why Sox2 increased in clustered mesenchymal cells; perhaps the clustering itself helps maintain stemness in some cases. In support of this, , Zhou *et al .,* (2017), found that nanog, a typical stem cell marker to be increased in clustered mesenchymal stem cells (Zhou, et al., 2017). In the same light, Chen *et al.,* (2019), showed that spheroid cultures of compact bone derived cells increase Sox2 and nanog as compared to when in monolayer cultures. However, they later found that when cultures are prolonged the expression gets downregulated (Chen, et al., 2019), suggesting that they could be differentiating. This perhaps suggests that clustering and stem cell state is a time dependent process.

4.2 Are human keratinocytes from glabrous skin (foreskin) able to dedifferentiate and express hair follicle stem cell marker?

Previous investigators have demonstrated that intact foreskin (glabrous) epithelium is able to be induced to form hair follicle-like structures when stimulated by inductive mesenchymal cells. Furthermore, hair-specific keratin markers (keratin 71 (inner root sheath); keratin 31 (hair cortex) keratin 14 (outer root sheath) were detectable by immunofluorescence in all the layers of the induced hairs (Ferraris, et al., 1997; Higgins, et al., 2013). This encouraging result indicates that in glabrous skin, there are bipotent stem cells that are able to be induced for form hair follicles.

The aim of the present study was to attempt to determine whether cultured keratinocytes from foreskin can be activated to express genes specific to hair follicles. A brief consideration of the different keratins is necessary before discussing the results obtained (Table 1). The difference between skin epidermis and the hair follicle epidermis is that the hair follicle is composed of 3 distinctive cell layers arranged concentrically as opposed to stratified layers of the epidermis (Figure. 3 in chapter 1). The basal layer of the epidermis which forms an undifferentiated region of the epidermis contains stem cells which express K5, K14 and K15. These keratins become downregulated in the differentiating suprabasal cell layers, switch on the differentiation-type keratins, K1 and K10 (Kirfel, et al., 2003) (Figure 3). The basal layer of the outer root sheath in the hair follicle also express K5, K14 and K5 in

addition to K19. In contrast to the epidermis that expresses K1 and K10 in suprabasal layers, the suprabasal layer of the outer root sheath express K6, K16 and K17. This information indicates that there are some important differences between the epidermis and the hair follicle as since they have different keratin expression profiles (Moll, et al., 2008).

In the present study, before exploring the different keratins in the cultured keratinocytes, it was first asked whether the cultured human foreskin keratinocytes as grown in this study do express stem cell markers. The early passage primary keratinocytes displayed epithelial stem cells properties by rapidly adhering to the plate surface forming keratinocyte colonies and expressing high levels of basal cell layer marker, K5 and low levels of differentiation-type marker, K10.The findings are somewhat line with those of Zhang *et al.,* (2012), where they showed that bulge hair follicle stem cells rapidly attached to the plate surface and highly expressed basal layer markers, K14 and alpha-6 integrin together with epithelial cell marker p63 and bulge stem cell marker, K15. To sum it up, these are cells that form hair follicles when co-transplanted with hair inducing dermal cells in nude mice (Zhang, et al., 2012).

As described in chapter 1, hair follicle morphogenesis is initiated by reciprocating signals between the epithelial and mesenchymal. This study explored the manner in which keratinocytes respond to mesenchymal cell factors.
To do this, keratinocytes were co-cultured with neonatal mouse mesenchymal cells, taking advantage of the size of the inserts that came with the cluster plates. The inserts had a diameter of 1.2 cm and the cluster plate diameter was 3.5 cm leaving approximately 1 cm area at the edges. Thus, the keratinocytes at the centre of the plate were directly below the mouse mesenchymal cells, while those at the edge of the plate were further away. This most likely created a “gradient effect, with cells at the centre receiving higher doses of factors than those at the edge. In line with this gradient hypothesis, the keratinocytes in the middle of the cluster had a higher cell proliferation rate than those of the periphery. These results are similar to some extent to was reported by Limat *et al*., (1993), where they found that clonal growth of outer root sheath keratinocytes on the insert increased when mesenchymal cells were located on the underside as compared to when on the dish bottom. This is because the factors from the underside of the insert are received immediately by the outer root sheath as compared to those at the base of the cluster plate which are additional separated by the medium (Limat, et al., 1993). This explains why the keratinocytes at the edges did not form more colonies since they received a lower dosage of secreted factors.

The ability to form keratinocyte colonies is a feature that is characteristic of *in vitro* epithelial stem cells (Oshima, et al., 2001). The results suggest that soluble factors from mesenchymal cells are capable of dedifferentiating keratinocytes. Keratinocytes that received signals from mesenchymal

cells showed an increased colony formation efficiency, as compared to those at the edges that received a lower dosage, indicating that they got undifferentiated in response to the factors they received. This result is consistent with studies by Limat *et al.,* (1993), and El-Ghalbzouri *et al.,* (2002), where they showed that cultured keratinocytes in the absence of feeders have low proliferative capacity and colony formation efficiency. They also showed that when the dosage of factors was increased by increasing the number of mesenchymal cells *in vivo* (El-Ghalbzouri, et al., 2002) and *in vitro* (Limat, et al., 1993), resulted in increased keratinocyte proliferative capacity and colony formation efficiency, respectively. This shows that keratinocytes can be stimulated to proliferate and also maintain undifferentiated state maintained by factors from the mesenchyme cells. Though not addressed in the present study, it is important to note that a number of factors have been identified that influence keratinocytes. Mesenchymal cell secreted factors include but not limited to, Wnt3a, transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), insulin growth factor (ISGF), ectodysplasin (Eda), sonic hedgehog (SHH) and bone morphogenetic protein (BMP) and they have been shown to be responsible for epithelial cell proliferation, follicular differentiation among other functions (Stenn & Paus, 2001; Kamp, et al., 2003; Inoue, et al., 2009; Won, et al., 2012). Also, of particular interest and relevance, Kim *et al*., (2018) have shown the exomes carrying factors are secreted from mesenchymal stem cells obtained from iPS cells and that these factors carrying exomes stimulate the proliferation of HaCaT keratinocytes.

In the present study, it was not possible to continue doing further hair follicle induction marker analysis of co-cultured keratinocytes because of significant problems related to their culture and expansion (see Technical considerations below). Therefore, the final set of experiments made use of the immortalised HaCaT cells, which has previously been shown to be able to differentiate into skin *in vitro* and *in vivo* (Boukamp, et al., 1988; Breitkreutz, et al., 1998; Schoop, et al., 1999).

In the first experiment the HaCaT cells were seeded at 10 x 10^4 in 12-well cluster plate with mesenchymal on the insert. Both β-catenin and the downstream signalling pathway gene, NF-KB expression levels were increased in the HaCaT cells as compared to controls without mesenchymal influence. The differentiation-type marker, K10 and basal cell layer marker, K5 gene expression levels were also increased in co-cultured HaCaT cells. The results suggested that neonatal mouse mesenchymal cells influenced HaCaT cells to activate the Wnt/β-catenin signalling pathway.

The findings are consistent to what was found by Pearton *et al.,* (2005) where dermal signals increased β-catenin and Lef1 protein levels in the basal layer of the epidermis. The findings are also in line to that of Chan, *et al.,* (2015) who found that outer root sheath keratinocytes cocultured with rat vibrissae passage 3 dermal papilla cells had increased β-catenin levels. In addition, differentiation makers, K1 and K14 and hair follicle specific markers including K6, K16, K17 and K75 (Table. 1 in chapter 1 for reference) were also upregulated (Chan, et al., 2015), and also with was found by Roh

et al., (2004), where human hair matrix keratinocytes co-cultured with human dermal papilla cells showed increased β-catenin expression levels at day 4 as compared to when keratinocytes are co-cultured with 3T3-J2 fibroblasts.

In the second experiment the number of HaCaT cells was reduced to 5 x 10^4 to avoid cells reaching confluency prematurely (and thence leading to differentiation). However, the results showed that β-catenin and K5 were slightly downregulated in HaCaT co-cultures. The downregulation of basal cell layer marker, K5 contrasted what Colombo *et al.,* (2017) who found that HaCaT cells expressed high levels of basal cell layer marker, K14 at 80% confluence as compared to overconfluent cells. However, the differentiation-type marker, K10 expression level was in line to what was discussed above (Colombo, et al., 2017).

As an additional approach, this study also explored whether keratinocytes could respond to Wnt3a-conditioned medium and upregulate expression of markers associated with early hair follicle morphogenesis. As was described in chapter 1, one of the Wnt/β-catenin signalling pathway molecules, β-catenin is expressed in the cytoplasm and membranes of hair placodes formed in basal cell layer epithelium of mice at embryonic day 14.5 (Ridanpaa, et al., 2001). This indicates that the canonical Wnt pathway, that gets activated by the binding of secreted Wnt3a to frizzled and LRP5 or LRP6 receptor complex on the cell membrane. This leads to the inhibition of glycogen synthase kinase-3 (GSK-3) and the accumulation and translocation of β-catenin into the nucleus which acts as a transcription factor together with TCF/LEF. This signalling is crucial for the initiation of hair follicle morphogenesis (Fig. 5 B in chapter 1) (Takahashi, et al., 2011).

The decision to use Wnt3a conditioned medium in the present study was motivated by a study conducted by Brown A (2017) 'Generating hair follicle inductive dermal papillae cells from adipose derived mesenchymal stem cells' (Master of Science in Medicine) University of Cape Town, Cape Town**.** Where Wnt3a conditioned medium was used to evaluate whether the Wnt/β-catenin signalling pathway genes would be upregulated in human adipose derived mesenchymal stem cells thereby inducing them to adopt a phenotype closer to that of the hair follicle dermal papilla cells. The presence of Wnt3 protein in the Wnt3a producing cells conditioned medium was confirmed by western blotting prior to performing all the experiments. Semi quantitative results revealed that Wnt3a is expressed four times higher in Wnt3a producing cells than in controls (Brown, 2017).

Unexpectedly, β-catenin and NF-KB genes were slightly reduced and only Lef1 was increased in Wnt3a cultured human keratinocytes. The result was unexpected because Wnt3a was supposed to directly activate the Wnt/β-catenin signalling pathway leading to the upregulation of Wnt signalling molecules. The fact that Wnt3a treatment only occurred after 3 weeks of culture which is considerably late for treatment, keratinocytes could have already differentiated and become postmitotic. The reduced cell proliferation rate experienced when culturing these cells could be associated with the

fact that normal human keratinocytes are prone to premature differentiation and loss of sensitivity as compared to HaCaT cells which make them a less reliable choice to use for experiments (Micallef, et al., 2008). Perhaps future studies should follow Sobel *et al.,* (2015) protocols, where they suggested that in order to increase sensitivity, keratinocytes should be co-cultured with mesenchymal cells to delay differentiation alongside treatment with Wnt3a conditioned medium to activate the Wnt/β-catenin signalling pathway (Sobel, et al., 2015), or epidermal cells should be treated with 6-bromoindirubin-3-oxime (BIO), a chemical that activates the Wnt/β-catenin signalling pathway by inhibiting glycogen synthase kinase 3 (GSK-3), with or without mesenchymal cells, since it has been reported that Wnt3a protein is unstable and only provides a short-lived stimulus (Tuysuz, et al., 2017).

4.3 Technical difficulties and limitations

Even though the results revealed that intact mouse dermis express dermal papilla signature genes the accuracy of the results could be questions because the cells used are a diverse mesenchymal cell population (and not only hair follicle mesenchymal clusters). In this study cell sorting was not done to separate cells that have hair follicle induction capacity from other dermal cells as was done others (Sorrell & Caplan, 2004) It is recommended that future studies utilise anti-CD133 antibodies to separate hair inducing dermal cells from non-hair follicle inducing dermal cells using flow cytometry.

In addition, bearing in mind that Sox2 is expressed in specific mouse hair types (Driskell, et al., 2009), its expression doesn't give a clear indication of hair follicle induction capacity of all dermal cells.

Efficient keratinocyte culturing methods are recommended in future studies. In this study, keratinocyte attachment efficiency varied for each experiment conducted making it difficult to plan accordingly because this led to variable number of cells that can grow. Variation of keratinocyte attachment inconvenienced the timepoint of doing co-cultures and conditioned medium treatment. For efficient keratinocytes expansion keratinocytes should have been expanded on feeder layer mesenchymal cells from the start to maintain their stem cell properties and the plates should have been coated with extracellular matrix for quick adhesion to maximise the number of cells needed for experiments. In addition, the keratinocytes used in this study were not purified to separate epithelial stem cells from differentiated cells or slowly adhering cells.

To purify epithelial stem cells, two approaches should be employed:

1. Allowing keratinocytes to grow out from the tissue and purification by a two-step trypsinisation procedure. This procedure takes advantage of the tight adhesion character of epithelial stem cells. The cells are first treated with a digestion solution at room temperature for 3-5 minutes, to allow the contaminating fibroblasts and differentiating epithelial cells to dissociate. The epithelial stem cells are then dissociated by incubating the remaining cells at 37℃ for 5 minutes (Zhang, et al., 2012).

2. Seeding epithelial cells on collagen IV coated plates and selecting cells that rapidly attach in the first 5 minutes of culture, because these cells have been found to express high levels of stem cell marker, B1-integrin and low levels of differentiating-type marker, involucrin; and they have been shown to have a high proliferative ability (Dallaglio, et al., 2013).

4.4 Future work and concluding remarks

This study demonstrates that neonatal mouse mesenchymal cells have hair induction capabilities, due to the expression of dermal papilla signature genes in intact dermis even though *in vitro* propagation perturbs this characteristic. It was very encouraging to find that dermal papilla signature genes can be restored in spheroid cultures since spheroid cultured mesenchymal cells are naturally akin to native dermal papillae. This opens that way to a very accessible method for studying the relationship between cause and effect of clustering and expression of stem cell markers and synthesis of signalling molecules including exomes (Kim, et al., 2018).

Having obtained positive results in HaCaT-mesenchyme co-cultures it shows that although the neonatal mouse mesenchymal cells have hair follicle induction capabilities, the , HaCaT cells cannot be used in human clinical trials because of their unregulated proliferation potential. Despite the technical difficulties experienced when culturing keratinocytes, co-culture results showed that keratinocytes require mesenchymal cell support for efficient expansion because they able to form colonies, a characteristic feature of epithelial stem cells *in vitro*. Perhaps future research should focus on optimising keratinocyte culture methods to generate competent keratinocytes that are capable of follicular differentiation.

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